Challenging and Emerging Pathogens in Cystic Fibrosis

Challenging and Emerging Pathogens in Cystic Fibrosis

Uitdagende en Opkomende Pathogenen bij Cystic Fibrosis

met een samenvatting in het Nederlands

Design: BUROPONY
Print: Platform P
ISBN: 978-90-393-5682-1

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Printing of this thesis was kindly supported by:
Bayer HealthCare Pharmaceuticals, B.Braun Medical BV, Forest Laboratories BV, Galapagos BV, Gilead Sciences Netherlands BV, GlaxoSmithKline, Hubbub, J.E. Jurriaanse Stichting, Novartis Pharma BV, Phadia BV, Stichting Kind en Afweer, Teva Pharmaceuticals Europe BV, Wilhelmina Children's Hospital, Zambon Nederland BV.

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 17 november 2011 des middags te 4.15 uur

door

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geboren op 28 april 1981 te Woerden

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Chapter 1

INTRODUCTION

CYSTIC FIBROSIS AIRWAY DISEASE

Cystic Fibrosis is a common genetic disorder, with an overall birth prevalence of 1 in 4750 live births in the Netherlands[1]. The genetic defect results in no or non-functional Cystic Fibrosis Transmembrane Regulator (CFTR) protein, causing defective transepithelial electrolyte transport. The main clinical manifestations are in the respiratory tract where it causes chronic inflammation and infection, and in the gastrointestinal tract where CF can lead to pancreatic insufficiency, meconium ileus and intestinal obstruction. Where in early days CF used to be fatal in children due to gastrointestinal manifestations, with current therapies for correction of gastrointestinal function life expectancy has increased and lung disease caused by chronic inflammation and infection has become the most important cause of morbidity and mortality[2;3]. Whether inflammation occurs primarily in the cystic fibrosis lung or only secondarily following bacterial infection is not entirely clear. A recent study in patients who were diagnosed through newborn screening has shown that already at the age of three months signs of structural lung disease can be seen on CT scans, and inflammation and bacterial infection can be present, despite the absence of respiratory symptoms[4]. Furthermore, levels of neutrophils, proinflammatory cytokines and elastase activity in uninfected infants were higher than expected in healthy infants. These data suggest that inflammation can precede bacterial infection.

Exactly how the defective CFTR protein results in pulmonary inflammation and infection with specific pathogens remains to be elucidated but several mechanisms have been described. The defective transport of Cl^- and $HCO3^-$, along with Na^+ hyperabsorption, causing depletion of pericellular airway liquid and decreased ciliary transport of pathogens, has long been thought to be the cause of CF lung disease[5]. However, a recent study using a $CFTR^{-/-}$ porcine model showed that CF lung pathology occurred in the absence of Na^+ hyperabsorption and depletion of pericellular airway liquid volume, suggesting an important role for defective anion transport[6]. Furthermore, hypertonicity of the airway surface liquid (ASL) is thought to result in less effective antimicrobial peptide activity[7], thus supporting pathogen colonization. Since many hypotheses exist, a multifactorial model is most likely to be at the base of cystic fibrosis lung disease.

CHALLENGING PATHOGENS

The respiratory secretions of CF patients harbor many pathogens of which several play an important role in CF lung disease. With the advent of new

detection techniques and the increased longevity of CF patients, new pathogens have emerged in the setting of CF lung disease and it is to be expected that this process will continue. Figure 1 shows the most important pathogens and the age-specific prevalence in CF patients of the Utrecht CF center in 2009 (data on file). The figure shows that the prevalence of respiratory pathogens changes with age; where *Haemophilus influenzae* and *Staphylococcus aureus* are important bacterial pathogens in childhood, *Pseudomonas aeruginosa* prevalence increases with age, chronically infecting 60-70 % of adult patients. *Aspergillus fumigatus* prevalence also increases with age, peaking in early adulthood. The challenges that come with these latter two pathogens will be discussed in this thesis.

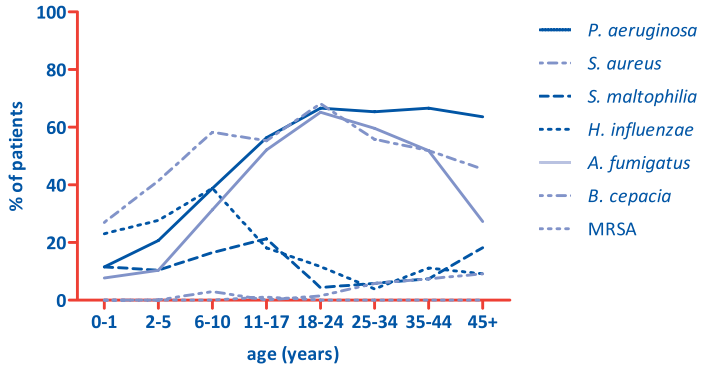

Figure 1 Age specific prevalence of respiratory organisms in 377 CF patients of the Utrecht CF center in 2009.

As mentioned before, decreased ciliary transport, depletion of pericellular airway liquid and reduced antimicrobial peptide activity probably support pathogen colonization in CF. Why CF patients become colonized by these pathogens in particular is not completely clear. Reasons for the early presence of *S. aureus* and *H. influenzae* are not clear, but these pathogens are also often found in throat cultures of healthy children[8] especially after viral infections or antibiotic therapy[9]. The presence of *A. fumigatus* generally occurs at a later age, and epithelial damage caused by bacterial pathogens is thought to facilitate its colonization[10]. The propensity for CF patients to become infected with *P. aeruginosa* specifically is the subject of ongoing research. Together with the above mentioned changes resulting in abnormal mucus, several other factors are suggested to play a role. Increased expression of asialo GM1 on epithelial cells resulting in enhanced binding of *P. aeruginosa*[11] and the finding that CFTR functions as an epithelial cell receptor for *P. aeruginosa* have been proposed[12].

Recently, ceramide accumulation in respiratory tract cells, leading to increased cell death, has been suggested to contribute to susceptibility by serving as adhesion factors for *P. aeruginosa*[13].

In this thesis we chose to focus on *P. aeruginosa* and *A. fumigatus*. *P. aeruginosa* is still the most prevalent pathogen in CF and although some progress has been made in research on eradication of *P. aeruginosa* and on the treatment of *P. aeruginosa* associated lung disease progression, there are still many challenges that remain. Furthermore, the more intensive treatment of *P. aeruginosa* and the increased life span of CF patients have created opportunities for other pathogens to emerge or become more prevalent. An increase in the prevalence of *A. fumigatus* in CF patients has been reported and with these reports a possible role in CF lung disease has been suggested, which could have implications for treatment strategies. In this thesis we will further investigate some of the challenges that come with these two pathogens.

PSEUDOMONAS AERUGINOSA

PATHOGEN CHARACTERISTICS

P. aeruginosa, is a ubiquitous Gram-negative rod that is widely distributed, and can be found in nature, where it is recovered from aqueous habitats including surface water, soil, sewage and plants[14]. The ability to thrive in such diverse environments is due to its large genome, which allows for genetic flexibility[15].

P. aeruginosa possesses an array of virulence factors that contribute to its pathogenetic potency. An important factor is the presence of surface components like pili and flagella, which are important in the initial infection. Another important determinant of virulence is the type III secretion system, which enables *P. aeruginosa* to inject effector proteins into host cells[16], playing a role in acute infections. The exact role of quorum sensing (QS) in *P. aeruginosa* remains to be understood. It controls many virulence factors, including biofilm formation, which is essential in establishing long-term infection, although evidence for a continuing role in CF infections is also described[17].

CHRONICITY

The infection process of *P. aeruginosa* in CF has three phases: no infection, intermittent infection and chronic infection[18]. Once the infection has become chronic it is rarely possible to eradicate *P. aeruginosa*, but during the initial

phases of the infection there is a window of opportunity for eradication. The current management of the initial detection of *P. aeruginosa* is by use of intensive antibiotic therapy[19] for which the optimal treatment regimen has not been established. In a recent large trial, the use of inhaled tobramycin inhalation for 28 days was similarly effective as 56 days. After 27 months of follow-up 66% of patients were still free of *P. aeruginosa*[20]. Other treatment strategies have been investigated and show varying success rates. Randomized controlled trials are still needed to compare various treatment regimens.

The first acquisition of *P. aeruginosa* generally involves unique strains which are obtained from the environment and which are different from the isolates found in chronically infected CF patients[21]. The wild-type isolates are generally nonmucoid, virulent and susceptible to antibiotics[22]. During the chronic infection process however, *P. aeruginosa* is able to adapt to the lung environment by genetic and phenotypic changes[23]. Important changes include the acquisition of antibiotic resistance, loss of motility and the conversion from a non-mucoid to a mucoid, alginate-overproducing phenotype[24]. The mucoid phenotype offers protection against host defense mechanisms and it is associated with a stronger deterioration of lung function[25]. It is thought that *P. aeruginosa* evolves towards a less virulent phenotype, in which virulence factors (necessary to establish acute infections) are selected against[23]. However, a mouse study showed that late chronic isolates from CF patients were attenuated in their capacity for causing acute mortality but were still capable of establishing chronic infection and inflammatory changes in mouse lungs[26]. This would suggest that during chronic infection clones with adapted virulence are established, but the effect of such adapted isolates in CF patients needs to be investigated.

HIGHLY PREVALENT CLONES

It was generally thought that every patient acquires their own *P. aeruginosa* 'wild type' strain from the environment and that sharing of genotypically identical strains does not occur outside households or people who are in close contact with each other[27]. This view was disputed by later reports of the occurrence of genotypically identical strains that were shared between patients, suggesting transmission of *P. aeruginosa* between patients[28-32]. In many of these reports, the highly prevalent clones were associated with an unfavorable outcome, and a causal relationship was suggested. However, many of these studies were prompted by a sudden deterioration in clinical condition in a small group of patients who shared the same *P. aeruginosa* genotype and did not involve

large-scale assessment of *P. aeruginosa* population structure. Also, longitudinal effects of infection were insufficiently studied and confounding was not always taken into account.

The reports did nonetheless raise concerns on transmission of *P. aeruginosa* between patients and it prompted the implementation of new infection control policies[33]. In the Netherlands as of 2005 the infection control policy for CF patients includes individual segregation of patients in both the in- and outpatient department[34]. Furthermore, the organizing of summer camps for CF patients has been discontinued and physical contact between patients outside the hospital is advised against.

Although reports from several other countries had described the occurrence of highly prevalent clones, it was not clear whether such a clone was also present in the Dutch CF population. Brimicombe et al. showed that during a summer camp for CF patients in 2001 many patients shared a strain that was similar upon typing, already suggesting a highly prevalent clone in the Dutch CF population[35]. Transmission during that particular camp was probable in 3 cases, which further supports the notion of transmission during such camps. Between 2007 and 2008 a cross-sectional study performed in all cultured CF patients of Utrecht and The Hague showed the presence of a highly prevalent *P. aeruginosa* clone (designated ST406), infecting 15% of all patients and showing an age-dependent prevalence[36]. Whether infection with this clone, as has been suggested for other highly prevalent clones, is associated with an unfavorable clinical outcome in the Dutch CF population has not yet been investigated and is an important aim of this thesis.

ANTIBIOTIC RESISTANCE

Although CF patients are treated with repeated long-term oral, intravenous and aerosolized antibiotics *P. aeruginosa* is rarely eradicated once the infection has become chronic, but bacterial loads can be decreased by using aminoglycosides, ß-lactam antibiotics and fluoroquinolones[37]. This suggests that the organism resides in the lungs under continuous antibiotic pressure, which raises concerns on the emergence of antibiotic resistance[38]. Several factors contribute to the resistance of *P. aeruginosa*, including its genetic capacity to express a wide repertoire of resistance mechanisms and its ability to become resistant through mutations in chromosomal genes which regulate resistance genes[39]. Additionally, *P. aeruginosa* can acquire resistance genes from other organisms via plasmids, transposons and bacteriophages. Furthermore,

the biofilm mode of growth in the CF lung confers resistance to eradication by antibiotics[40].

Risk factors for antibiotic resistant *P. aeruginosa* in CF patients include the use of intravenous (IV) antibiotics, which are administered during acute exacerbations[41]. The risk associated with the use of inhaled antibiotics is not yet fully known. The trials investigating the efficacy and safety of the use of inhaled tobramycin have shown increases in MIC values over time, but this did not correlate with therapeutic success[42]. In another cohort study however, the use of inhaled tobramycin was an independent risk factor for acquisition of multiple antibiotic resistant *P. aeruginosa* (MARPA). However, as this study evaluated the risk factors for MARPA, and thus included strains resistant to quinolones, but did not take into account the use of oral quinolones, it is difficult to evaluate the risk associated with specific types of antibiotic[41]. The contribution of inhaled antibiotics to the risk of resistance in the long term thus remains to be established.

CO-INFECTIONS

There is increasing evidence that respiratory virus infections play a role in CF exacerbations by facilitating bacterial colonization. In clinical studies, the frequency of viral infections was similar in CF patients when compared to healthy controls, but these infections were more frequently associated with lower respiratory tract symptoms[43]. Furthermore, epidemiological studies showed a seasonal pattern of first *P. aeruginosa* acquisition[44] and development of chronic *P. aeruginosa* infection and a rise in *P. aeruginosa* antibodies with RSV infections suggesting the influence of respiratory viruses[45]. The possible interaction between respiratory viruses and bacteria was suggested from in vitro studies, showing increased adherence of *P. aeruginosa* to airway epithelial cells during viral infection and direct binding between *P. aeruginosa* and RSV[46;47]. Studies in mice also showed decreased clearance of *S. pneumoniae* from the lungs of RSV-infected mice[48]. In another mouse model of RSV infection, an enhancing effect on *S. pneumoniae* bacteraemia was found upon simultaneous intranasal inoculation[49]. In this model, the enhancing effect of simultaneous RSV infection was abrogated upon administration of heparin, which interacts with the RSV glycoprotein G and inhibits RSV infectivity, showing that the increased binding of the bacteria was through the glycoprotein G[49]. Together, these observations suggest a possible facilitating role for respiratory viruses on *P. aeruginosa* colonization, which could offer new possibilities for preventive measures.

In this thesis the following research questions regarding *P. aeruginosa* will be addressed:

- Does simultaneous infection with RSV facilitate acute colonization with *P. aeruginosa* in a mouse model and can this effect be abrogated by heparin treatment?
- Is infection with a highly prevalent *P. aeruginosa* clone associated with a less favorable clinical outcome?
- Does transmission of *P. aeruginosa* occur during a one-day open-air event for CF patients?
- Does infection with the highly prevalent clone persist?
- Is the use of tobramycin inhalation associated with an increased risk of aminoglycoside resistance?

ASPERGILLUS FUMIGATUS

PATHOGEN CHARACTERISTICS

Aspergillus fumigatus is a ubiquitous fungus that is commonly found in house dust and decomposing organic matter[50] and it is the most common filamentous fungus cultured in CF. The prevalence of this fungus in respiratory secretions of CF patients varies, which is related to topographic location and especially to detection methods used. Reported prevalence from single or multicenter studies ranges from 6 to 57%[10]. Similar to chronic *P. aeruginosa* isolates, CF patients are colonized with environmental isolates but persistent clones have been described[52].

CLINICAL IMPLICATIONS IN CF

The most specific clinical manifestation of *A. fumigatus* in CF is Allergic BronchoPulmonary Aspergillosis (ABPA), which is characterized by pulmonary infiltrates and production of IgG and IgE antibodies specific for *A. fumigatus* as well as a rise in total IgE[53]. ABPA prevalence varies between 2 and 8% and it was associated with a more severe lung function decline[54]. A possible negative effect on lung disease in the absence of ABPA was suggested by reports of patients colonized with *A. fumigatus* whose respiratory deterioration did not respond to antibiotics, but was brought to a halt by antifungal therapy[55]. A number of cohort studies have investigated the effect of *A. fumigatus* colonization in the absence of ABPA and found conflicting results regarding its effect on lung function decline[56-58]. It is important to further study this issue, because a negative effect of *A. fumigatus* on lung function could indicate the need for antifungal therapy in the case of *A. fumigatus* colonization.

In this thesis the following research questions regarding *A. fumigatus* will be addressed:

- What is the prevalence of *A. fumigatus* in CF patients of the Utrecht CF center?
- Is *A. fumigatus* positivity an independent risk factor for unfavorable clinical outcome?
- Is chronic infection with *A. fumigatus* associated with a stronger pulmonary function decline?

Chapter overview

In this thesis we give an overview of several emerging pathogens. Furthermore, by focusing on several determining factors associated with *P. aeruginosa* and *A. fumigatus* we try to elucidate whether and how these factors influence CF lung disease.

Chapter 2 gives an overview on the epidemiology, clinical impact and treatment of several challenging and emerging pathogens in cystic fibrosis.

Chapter 3 describes the effect of simultaneous RSV infection on the acute colonization with *P. aeruginosa* in a mouse model, and the effect of heparin on this co-infection model.

Chapter 4 presents the results of a cohort study on the prevalence and risk factors for *A. fumigatus* colonization and the longitudinal effect of colonization on lung function decline.

Chapter 5 presents the results of a study on the transmission of *P. aeruginosa* during a one-day open-air event on the beach for CF patients.

Chapter 6 describes the clinical characteristics associated with infection with the highly prevalent Dutch clone ST406 in the population of the Utrecht and the Hague CF centers.

Chapter 7 provides a longitudinal analysis of *P. aeruginosa* genotype persistence and clinical outcome over a 10-year period.

Chapter 8 examines the association between ST406 infection and the course of CF lung disease.

Chapter 9 describes the use of inhaled tobramycin and the associated risk of aminoglycoside resistance in *P. aeruginosa*, in a cohort of CF patients followed for up to 8 years.

Chapter 10 provides a general discussion of the studies presented in this thesis and discusses directions for future investigations.

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Chapter 2

CHALLENGING AND EMERGING PATHOGENS IN CYSTIC FIBROSIS

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PAEDIATRIC RESPIRATORY REVIEWS 2010 DEC;11(4):246-54.

Abstract

Cystic fibrosis (CF) lung disease is characterized by chronic inflammation and infection. Patients are predominantly infected by specific pathogens, of which Staphylococcus aureus and Pseudomonas aeruginosa are the most important. In recent years however there has been an increasing number of reports on potentially emerging and challenging pathogens like Stenotrophomonas maltophilia, non-tuberculous mycobacteria, highly prevalent P. aeruginosa clones, methicillin resistant Staphylococcus aureus and Burkholderia cepacia. Also, a role for viral infections in the pathogenesis of CF lung disease has increasingly been recognised. It is not always clear whether or how these pathogens influence the progression of CF lung disease and how they should be treated. In this review, the epidemiology and clinical impact of these pathogens is discussed. Furthermore, treatment strategies of these pathogens in a CF setting are reviewed.

Introduction

Patients with cystic fibrosis (CF) are predominantly infected by specific pathogens, of which *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most important. In recent years, there has been an increasing number of reports on potentially emerging and challenging pathogens including *Stenotrophomonas maltophilia*, non-tuberculous mycobacteria, highly prevalent ('epidemic') *P. aeruginosa* clones, Methicillin resistant *Staphylococcus aureus* and *Burkholderia cepacia* complex. Also, a role for viral infections in the pathogenesis of CF lung disease has been recognised.

Although some of these pathogens seem to become more prevalent, evidence on the clinical significance of their isolation is not always unequivocal. The nature of CF lung disease is multifactorial. Besides the presence of pulmonary pathogens, factors such as nutritional status, CF related diabetes and pancreatic insufficiency contribute to the progression of lung disease[1]. The effects of individual pathogens on a population level can therefore differ from the effects of pathogens in individual patients. Furthermore, it is not always clear whether a pathogen is truly emerging, or whether the increasing prevalence is related to enhanced detection using new molecular techniques and increased surveillance, as advocated in international guidelines. In this review, the epidemiology, clinical consequences and treatment of emerging or challenging pathogens are discussed.

The propensity of patients with CF to become infected with specific pathogens is only partially elucidated, and many factors play a role. Hypertonicity of the airway surface liquid (ASL) is thought to result in less effective antimicrobial peptide activity[2]. High viscosity of the periciliary liquid layer plays a role in decreased airway clearance. In addition, hyperproduction of abnormal mucus due to CFTR dysfunction adds to the reduction of functional ciliary transport of pathogens[3]. Another factor possibly contributing to the increased susceptibility specifically for *P.aeruginosa*, involves asialoganglioside I (aGMI) in the apical membrane. Increased expression of asialo GM1 on epithelial cells in CF has been reported to lead to enhanced binding of *P. aeruginosa*. The finding that CFTR is an epithelial cell receptor for *P. aeruginosa* has served as the basis for another hypothesis. This suggests that *P. aeruginosa* binding to CFTR results in internalization by epithelial cells, which are then shedded into the ASL[4]. In a recent study it was shown that in CFTR-deficient mice, increasing age leads

to an accumulation of ceramide in the respiratory tract, resulting in age-dependent pulmonary inflammation, death of respiratory cells and an increased susceptibility to severe infection [5]. The study suggested that increased DNA deposits due to cell death might serve as adhesion factors for *P. aeruginosa*. As there have been reports both supporting and contradicting the above described factors, a multifactorial model is most probable and the exact contribution of each factor needs to be studied further [4].

VIRAL PATHOGENS

For pulmonary exacerbations of chronic respiratory illnesses like asthma[6] and chronic obstructive pulmonary disease (COPD)[7;8] the importance of respiratory viruses has been well recognised. Studies report virus involvement in up to 85% of asthma exacerbations[6]. A role for respiratory viruses has increasingly been reported in pulmonary exacerbations in CF and associated lung function decline.

EPIDEMIOLOGY

Respiratory viruses that have been investigated include RSV, influenza viruses, adenovirus, rhinovirus, picornavirus and human metapneumovirus. Although studies differ in the detection methods used, they are consistent in reporting that the prevalence of respiratory virus infections is not different in CF patients when compared to healthy subjects[9-12]. In one prospective study, viruses were detected even less frequently in CF patients during episodes of acute respiratory tract illness. The authors attributed this finding to a lower daycare attendance in CF patients and not to CF disease specific factors[9].

CLINICAL IMPACT

Although some studies are impeded by inadequate detection of respiratory viruses, many report an association between respiratory virus infections and morbidity in CF[9;12-15]. In studies in infants and young children with CF, respiratory virus infections were associated with an increased risk of hospitalization[9;11] and prolonged lung function deterioration[9;11] compared to non-CF controls, showing that the virus-related morbidity is greater than in healthy controls. Van Ewijk et al. prospectively studied a group of 20 children with CF and 19 age matched controls during a 6-month winter period using PCR for virus detection. Although there was no difference in frequency of acute respiratory illnesses, nor in the upper respiratory tract symptoms, children with CF did have longer and more severe periods of lower respiratory tract symptoms[12].

These results stress the significance of respiratory virus infections and suggest that targeting respiratory viruses in new therapeutic strategies might improve prognosis.

There are several hypotheses explaining the enhanced effect of viral infections in CF patients. It has been suggested from in vitro and animal studies that the impaired innate host response in CF leads to higher virus replication[12;16;17]. Other than a direct effect of virus infections on pulmonary inflammation, an indirect role of respiratory viruses through interaction with pathogenic bacteria has also been proposed. Epidemiological observations showed that first acquisition[18] or the development of chronic *P. aeruginosa* infection coincided with respiratory virus infections[19], suggesting that viruses pave the way for *P. aeruginosa* colonization and infection. In vitro studies propose increased adherence of bacterial pathogens to damaged airway epithelial cells following virus infections as a possible mechanism[20]. Several strains of *P. aeruginosa* showed increased adherence in the presence of RSV and direct binding of *P. aeruginosa* to the virus in vitro, suggesting that RSV can act as a coupling agent between the bacteria and the host cells [21]. In an acute mouse model of *P. aeruginosa* and RSV infection, simultaneous challenge of mice with *P. aeruginosa* and RSV showed increased bacterial loads and more severe lung function changes, when compared to mice infected with bacteria alone[22]. These results support the notion of an enhancing effect of respiratory virus infections on *P. aeruginosa* infection and provide further explanation for the clinical observations of the effect of respiratory viruses in CF.

MANAGEMENT

Several prevention strategies aimed at respiratory virus infections have been investigated. Influenza vaccination is commonly recommended for CF patients, but randomised placebo-controlled trials are lacking. Studies have not been able to show clear improvements in clinically relevant outcomes[23] like lung function and number of hospitalizations. The use of antiviral agents against influenza has not been studied yet in CF patients.

The prevention of RSV infection by passive immunization with palivizumab, a monoclonal antibody, has been studied retrospectively and in one study children receiving palivizumab had a lower risk of hospitalization[24]. One randomized controlled trial has been performed[25], of which data were published in abstract form only. In this study 186 children were included who received monthly injections of either palivizumab or placebo during the

respiratory virus season. No significant difference in number of *P. aeruginosa* infections was found between the treatment and placebo group. It is difficult to draw any conclusions as to the clinical significance of the intervention, because pulmonary function and hospitalization for RSV infection were not assessed in this study. Further randomized trials are necessary to study the benefits of the costly palivizumab on prevention of serious exacerbations and associated decline in lung function.

Other possible preventive measures could be directed at the facilitating effect of viral infections on bacterial pathogens as has been shown from epidemiologic and laboratory data as described above. The prophylactic use of antibiotics during periods of viral infections, or during the respiratory virus season, are conceivable options to prevent the secondary colonization or infection with bacteria like *P. aeruginosa*. However, studies investigating this are lacking. Also more specific therapies aimed at the mechanisms by which viruses can interact with other pathogens might be developed once such mechanisms have been studied more extensively.

BACTERIAL PATHOGENS

STENOTROPHOMONAS MALTOPHILIA

S. maltophilia is a non-fermenting gram-negative rod, with an intrinsic resistance to broad-spectrum antimicrobial agents including carbapenems, leading to a limited array of treatment options. The bacterium can be isolated from nosocomial sources like central venous catheters, nebulizers and deionized water dispensers as well as from sources outside the hospital like water sources (rivers, wells) and from soil and plant environments[26;27]. *S. maltophilia* is an important nosocomial pathogen, infecting immunocompromised patients and those on ventilator support[26]. Infection with *S. maltophilia* is associated with endocarditis, sepsis, meningitis, peritonitis and soft tissue and wound infections[28], making it a challenging pathogen in the ICU.

EPIDEMIOLOGY

Reports on the prevalence of *S. maltophilia* in CF have shown conflicting results: some studies report an increase of *S. maltophilia* prevalence in recent years[29] and suggest that it is an emerging pathogen, whereas others report no trend in its prevalence[30-32]. Unlike the infection process with *P. aeruginosa*, many patients are only intermittently infected with *S. maltophilia*[30;33].The reported incidences of *S. maltophilia* in CF patients range from to 2-14%[29;30;34-38].

Table 1 displays the prevalence of *S. maltophilia* in the Utrecht CF center, and shows that the prevalence in our center has increased over the last 6 years. Although this is partly due to the increased culturing frequency, when taking into account the number of cultures per patient per year, the proportion of positive cultures still increased.

Similar to observations in non-CF patients, the emergence of *S. maltophilia* has been linked to the use of anti-pseudomonal antibiotics in CF patients. In a retrospective case-control study, Denton et al. found that in the years preceding the first isolation of *S. maltophilia*, colonized patients had received more oral ciprofloxacin and intravenous anti-pseudomonal antibiotics[39]. Similarly, upon evaluating the effect of intermittent inhalation of tobramycin on the microbiology of respiratory secretions[40], Burns et al. found that the use of oral quinolones was the only factor that significantly increased the risk of *S. maltophilia* isolation[34]. The authors suggest the selection of quinolone-resistant organisms by quinolone use. In non-CF patients the use of carbapenem antibiotics has been associated with isolation of the organism[41].

Table 1 Annual prevalence of *S. maltophilia*, MRSA and *B. cepacia* in patients seen at the Utrecht CF center

Year	Patients cultured	Cultures per patient	*S. Maltophilia*	MRSA	*B. Cepacia*
2002	208	2.3	3 (1.4%)	0 (0%)	1 (0.5%)
2003	237	2.8	7 (2.9%)	2 (0.8%)	2 (0.8%)
2004	295	3.1	20 (6.8%)	0 (0%)	2 (0.7%)
2005	311	3.4	22 (7.1%)	0 (0%)	2 (0.6%)
2006	323	4.6	34 (10.5%)	0 (0%)	5 (1.5%)
2007	329	5.1	33 (10.0%)	0 (0%)	7 (2.1%)

CLINICAL IMPACT

Clinical infections observed in non-CF patients suggest a potential pathogenic role for *S. maltophilia* in CF-patients. It has been reported that *S. maltophilia* negatively impacts CF lung function[42]. In a number of case-control and cohort studies, *S. maltophilia* positive patients did generally have decreased

pulmonary function, but during follow-up, which was mostly short, no clear difference in deterioration of clinical status was noted[30;32;35;38;39]. In a cohort study comprising around 20,000 CF-patients, Goss et al. showed that although there was a decreased lung function at the time of positivity for *S. maltophilia*, after adjusting for confounding factors there was no difference in lung function decline between positive and negative patients[31]. The authors had previously shown that in a similarly large cohort *S. maltophilia* positivity did not result in any difference in 3-year mortality[27]. This suggests that *S. maltophilia* is most likely a marker for more severe disease and that a negative impact on lung function decline has not been proven on a population level. In our experience however, in the case of a sudden deterioration associated with *S. maltophilia* a beneficial effect of treatment can be observed.

MANAGEMENT

In individual cases of acute worsening of pulmonary status and the concomitant presence of *S. maltophilia* from respiratory cultures, treatment seems warranted. Most data on the treatment of *S. maltophilia* come from case reports or in vitro studies[43]. San Gabriel et al. investigated isolates from 673 CF patients and studied the in vitro activity of ten antimicrobial agents against these isolates, also performing synergy studies[44]. Doxycycline was most active, inhibiting 80% of isolates. Also, colistin was proven to be more active than highly dosed gentamicin and tobramycin. Synergystic or additive activity was most profound with the use of trimethoprim-sulfamethoxazol plus ticarcillin-clavulanate and trimethoprim-sulfamethoxazol plus piperacillin-tazobactam with 65% and 64% of isolates sensitive[44]. Although these in vitro studies provide guidelines for treatment, they may not mimic the clinical situation in the CF lung and therefore controlled clinical studies are necessary to determine which antibiotic is most effective in a clinical setting.

NON-TUBERCULOUS MYCOBACTERIA

Non-tuberculous mycobacteria (NTM) are widely distributed in the environment, where they can be found in water sources (natural and treated) and in soil[45]. NTM species include *M. avium* complex, *M. kansasii* (these two organisms represent the most frequently encountered species) and *M. abscessus*. General criteria for diagnosing nontuberculous mycobacterial lung disease have been published[46]. Two clinical criteria are required: (1) pulmonary symptoms or nodular/cavitary opacities on chest radiograph or HRCT showing multifocal bronchiectasis with multiple small nodules and (2) other diagnoses should be appropriately excluded. Furthermore, one of the following microbiological

criteria is required: (1) positive sputum culture results on two separate occasions, (2) positive culture result from at least one bronchial wash or lavage or (3) mycobacterial histopathologic features in lung biopsy and positive culture from biopsy, sputum or wash.

The difficulty in culturing NTM from non-sterile body-sites lies in the fact that they are frequently overgrown by other bacteria or fungi present in the sputum, especially in CF patients. Therefore, digestion and decontamination procedures should be performed upon laboratory analysis.

EPIDEMIOLOGY

Over the past decades, non-tuberculous mycobacteria (NTM) have been reported in CF patients. The most common organisms are *Mycobacterium abscessus* and *Mycobacterium avium* complex (MAC) and the reported overall prevalence of NTM in CF patients ranges from 3.8% to 22.6%[47-49]. The difference in reported prevalences in literature could be explained by study design (point-prevalence versus longitudinal) and different culturing methods but could also reflect a difference in microbiological ecology[49]. As suggested previously, it is important to employ specific culturing techniques in order to determine the prevalence of NTM as these pathogens will not be detected using regular culturing techniques.

CLINICAL IMPACT

There have been several case reports and studies suggesting a relationship between adverse clinical status and NTM in individual CF patients[50;51] and other studies fail to show a clear association[52]. Not all studies evaluated decline of lung function and other clinical parameters though, making it difficult to study a causal relationship. In a large multi-center study, Olivier et al. prospectively studied the effect of NTM positivity on the decline of FEV_1 and found no significant difference in FEV_1 decline during the 15 months of follow-up[50]. Upon HRCT evaluation however, a difference was found in progression of HRCT findings, where patients with 3 or more positive cultures for NTM were more likely to show progression on HRCT compared to other patients[50]. Furthermore, in a recent retrospective study in a single CF center, significantly greater rates of lung function decline were found in patients with chronic *M. abscessus* infection over an 8-year period (chronic infection defined as 3 or more positive cultures over 3 or more quarterly visits)[53]. These studies suggest that, although NTM might not show a direct effect on lung function decline, significant increase of lung pathology is possible, warranting screening and

vigorous follow-up of patients with positive cultures for NTM, including the use of imaging studies to detect characteristic pulmonary changes.

MANAGEMENT

Based on the studies suggesting a significant clinical effect of NTM, close surveillance of CF patients seems warranted. Olivier et al. propose that CF patients with repeatedly positive cultures for NTM and concomitant characteristic HRCT changes should be monitored for progression of these HRCT changes[50]. Should the changes be suggestive for mycobacterial disease, or should they be progressive, specific antimycobacterial therapy is warranted.

Evidence for treatment of NTM using specific antimycobacterial agents stems mostly from studies in HIV/AIDS patients. For CF patients, in order to monitor the response to antimycobacterial therapy, it is of importance to ensure maximal treatment of other pathogens before specific antimycobacterial therapy is started, due to overlap with antimicrobial agents aimed at treating other CF pathogens. Recommended drug treatment for the most common NTM (MAC) is depicted in Table 2. Unlike the anti-TB medications, newer macrolides (clarithromycin and azithromycin) show much better correlation between in vitro susceptibility and treatment response[54].

Table 2 Recommended treatment regimen for *M. avium* complex (MAC) (adapted from Griffith et al.[46])

	Initial therapy for nodular/ bronchiectatic disease	**Initial therapy for cavitary disease/ advanced or previously treated disease**
macrolide	clarithromycin 1000 mg 3x/week or azithromycin 500-600 mg 3x/week	clarithromycin 500-1000 mg/d or azithromycin 250-300 mg/day
ethambutol	25 mg/kg 3x/week	15 mg/kg/day
rifampicin	rifampin 600 mg 3x/week	rifampin 450-600 mg/day
IV **aminglycoside**	none	streptomycin or amikacin (or optionally none in cavitary disease)

For *M. abscessus*, treatment strategies to eradicate the organism from the sputum are lacking. *M. abscessus* isolates are resistant to the standard antituberculous agents and therefore treatment options are limited. Therapy with macrolides alone is not sufficient and combination therapy with amikacin and cefoxitin or imipenem for 2-4 months can improve clinical status but this comes with high cost and morbidity[46]. Linezolid, belonging to a newer drug class (oxazolidinones) shows some potential. Of *M. abscessus* isolates, around 50% are susceptible or intermediate in susceptibility in vitro to linezolid[46]. Cremades et al. investigated two CF isolates of *M. abscessus* for the in vitro sterilizing capacity of several antibiotic agents and found good activity exhibited by clarithromycin in combination with linezolid, suggesting that the addition of linezolid might be useful[55].

PSEUDOMONAS AERUGINOSA — HIGHLY PREVALENT CLONES

P. aeruginosa is a Gram-negative rod that has a widespread presence in the environment. It is the most important pathogen in CF. During long-term infection *P. aeruginosa* can develop numerous mutations in its bacterial genome. Through this process, the phenotypic characteristics gradually alter after primary infection. The infection process in CF patients generally consists of three phases: no infection, intermittent infection and chronic infection[56]. Subsequently, the development of mucoid *P. aeruginosa*, which is a mutant phenotype of *P. aeruginosa*, occurs. The mucoid phenotype produces exopolysaccharides/alginate and confers resistance to antibiotics and the host immune system, making it difficult to eradicate. Since the early nineties, the emergence of highly prevalent clones of *P. aeruginosa* has been described and the prevention of such clones has become a target of infection control policies[57].

EPIDEMIOLOGY

The prevalence of *P. aeruginosa* in CF increases with age, chronically infecting up to 80% of adult patients[58]. With studies of infants diagnosed through CF newborn screening, it has become clear that *P. aeruginosa* can be isolated already at an early stage of the disease. In a recent study from Australia, 57 infants diagnosed through newborn screening underwent bronchoalveolar lavage and chest CT. Although respiratory symptoms were absent in 48 (84%) patients, in 12 (21%) patients lung disease with bacterial infection was observed and in 3 infants *P. aeruginosa* could be detected[59]. This suggests that the pathogen is already present in the lower airways of infants with CF before chronic infection becomes apparent through sputum or oropharyngeal culturing,

Until recently, the general view of the infection process with *P. aeruginosa* was that each CF patient carried his/her own unique strain that is mainly acquired from the environment[60-62]. Transmission of *P. aeruginosa* strains was considered to occur only between patients who had prolonged contact, i.e. siblings[63] or patients who visited holiday camps together[64]. This view has been disputed by reports of *P. aeruginosa* clones that are shared between patients, suggesting an important role for patient to patient transmission[65-69] as these clones are not isolated from the environment. Strong evidence for patient to patient spread was reported from the CF center in Liverpool, where molecular fingerprinting techniques identified an outbreak of a β-lactam resistant *P. aeruginosa* strain (referred to as the Liverpool Epidemic Strain, LES)[65]. Later, similar reports of unrelated CF patients sharing *P. aeruginosa* strains, identified using molecular techniques, occurred from centers in Australia, Europe and other centers in the UK[66;67;69;70]. Recently, a study of two large CF centers in the Netherlands showed the presence of two highly prevalent clones that were not genetically related to other published clones[71]. The presence of the clones did cluster by age, suggesting the spread of the clone during social contact or holiday camps.

CLINICAL IMPACT

In some cases, the detection of highly prevalent clones was prompted by a sudden deterioration in a group of CF patients or the increased occurrence of *P. aeruginosa* strains with a certain resistance pattern[65;67]. It has been hypothesized that such clones have specific characteristics that enable them to either be more virulent or to be easily transmissible and some of the clones have been found to have specific phenotypic characteristics[72;73].

Whether infection with a highly prevalent clone is associated with an unfavorable clinical outcome has been investigated in several centers. An overview of such studies is given in Table 3. Most of these studies show an association between the presence of a highly prevalent clone and unfavorable clinical outcome[67;74-77]. However, many of these studies are cross-sectional or report only on a small group of patients. In a longitudinal case-control study of 12 patients infected with the LES, Al-aloul et al. showed a significantly greater annual loss of lung function and deterioration of nutritional status[75]. In contrast, Jones et al. recently reported a longitudinal study which included 80 patients[78]. Patients were followed for 8 years and designated as either being infected with a highly prevalent clone (including the LES) or with a sporadic clone. No differences in baseline characteristics and also no differences in annual change

in lung function or BMI were observed. The patients infected with a clone did have significantly increased numbers of IV antibiotic treatment. The authors suggest the possibility of successful aggressive antibiotic treatment in the patients with a clone, which could have prevented a more rapid deterioration of lung function. As to the cause of increased treatment burden, this could be due to increased treatment requirements in the patients carrying the clone, but it could also be due to the physicians' knowledge of the patients' infection status. Because of these conflicting results further study into the longitudinal effect of chronic infection with such highly prevalent clones seems warranted.

MANAGEMENT

The emergence of the highly prevalent clones has lead to segregation measures aimed at controlling the spread of such clones. Infection control measures include cohort segregation, which divides patients by *P. aeruginosa* infection status or genotype of their *P. aeruginosa* strain, but in some CF units complete individual segregation has been implemented. Also, social contact between CF patients is discouraged and the organisation of summer camps has been discontinued. The effects of these segregation measures have been reported by some centers. In Australia, the prevalence of a clone found in the CF unit of Brisbane was significantly reduced by the implementation of cohort segregation[77]. In a Danish CF center, the presence of a highly prevalent clone was greatly reduced after implementation of segregation[79]. Since the measures implemented come with a high psychosocial burden, continued studying of the benefits of infection control measures together with the clinical effects of transmissible clones is warranted.

Maintenance therapy for treatment of *P. aeruginosa* consists of inhaled antibiotics, accompanied by oral macrolide therapy[80]. As some of the reported clones are characterized by a specific antibiogram, treatment of infection with such clones is adjusted according to their resistance profile. The LES is characterized by resistance to ceftazidime, but it is susceptible to tobramycin and ciprofloxacin[65]. It is stressed that early aggressive antibiotic therapy using inhaled antibiotics either as monotherapy (tobramycin)[81] or inhaled antibiotics in combination with oral antibiotics (colistin and ciprofloxacin)[82] is important to prevent or delay chronic *P. aeruginosa* infection. It has been shown that early treatment can slow the decline of pulmonary function after initial colonization with *P. aeruginosa*[82].

METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

EPIDEMIOLOGY

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a pathogen that is imposing an increasing problem in for instance intensive care units, where it is associated with morbidity and mortality. The increasing prevalence of this pathogen in the general population is mirrored in CF patients. In the US CF population the prevalence of MRSA has increased markedly in the past decades, from 2.1% in 1996 to 22.6% in 2008[83;84]. Similarly, in adult patients in a UK CF center, the prevalence increased from 1 % in 1985 to 6% in 2005[85]. In the Utrecht CF center, the prevalence of MRSA is very low (Table 1), which is similar to the low prevalence of MRSA in other patients in the Netherlands, where MRSA infection control policies consist of pre-emptive isolation of patients who are considered at high risk of MRSA carriage, until the absence of MRSA is proven[86].

CLINICAL IMPACT

With the increasing prevalence of MRSA in CF patients there have been many studies investigating the clinical significance of this pathogen. Several cross-sectional studies show conflicting results of whether an association exists between MRSA and decreased lung function[87;88]. Ren et al. reported of a large cross-sectional study using data from an epidemiologic study (ESCF), in which patients with MRSA had a lower FEV_1% predicted compared to patients infected with methicillin sensitive *Staphylococcus aureus* (MSSA)[87]. Several small studies have investigated the effect of MRSA longitudinally. Miall et al. conducted a case-control study including 10 children with MRSA and 18 controls, showing no difference in lung function change[89], whereas Thomas et al. studied 23 patients with MRSA and 69 controls and found that lower FEV_1 was a risk factor for MRSA acquisition[90]. In this study however, pulmonary function decline was not studied, but there appeared to be no difference in mortality between controls and MRSA positive patients. A large longitudinal study was performed by Dasenbrook et al., in which they evaluated 1732 patients who developed new persistent MRSA infection and were followed for 3.5 years[83]. The authors demonstrated that even after adjustment for confounders, the rate of FEV_1 decline is higher in the persistently infected patients when compared to patients with only negative cultures, but this was only of clinically relevant magnitude in those aged 8-21. The authors also showed that in persistently infected patients, the rate of lung function decline in the period after first detection of MRSA was significantly greater than before[83]. These results imply that at least between the ages of 8 and 21, persistent MRSA infection negatively impacts lung function.

Table 3 Studies reporting of clinical impact of highly prevalent clones

	Armstrong 2002[67]	Jones 2002[66]	Al-Aloul 2004[75]	O'Carroll 2004[76]	Griffiths 2005 [77]	Jones 2010[78]
study type	cross-sectional	cross-sectional	case-control	cross-sectional	retrospective cohort	longitudinal cohort
clone	Australian Epidemic Strain 1 (AES-1)	Manchester	Liverpool Epidemic Strain (LES)	Australian Epidemic Strain 2 (AES-2)	Australian Epidemic Strain 1 (AES-1)	LES + Manchester
typing method	PFGE, RAPD	PFGE, pyocin typing	strain genotyping	PFGE	PFGE	PFGE
number of *P. aeruginosa* positive patients (% with clone)	118 (55%)	78 (28%)	24 (50%) (case-control study)	100 (39%)	149 (27%)	80 (35%)
patient selection	Sep-Dec 1999; patients seen for routine visit	2000: Inpatient i.v. ab treatment	LES neg patients 1998-2003 matched with LES+ patients (matched for lung function, BMI, age, sex, medication, subdiagnoses)	Dec 2001- juli 2002: per center first 50 patients with PA+ sputum culture	2002: patients seen for routine visit	1999: patients requiring in-patient treatment
proportion of total population sampled	samples from all 152 sputumproducers (49% of all patients)	?	?	100 of 283 patients (35% of all patients)	149 samples from sputumproducers (51% of all patients)	samples from all patients

MANAGEMENT

Treatment options for MRSA in CF have been investigated in small clinical studies. The published treatment regimens are shown in Table 4. In a retrospective study, Solis et al. described the results of an eradication protocol consisting of oral/topical application of vancomycin for carriers and nebulized vancomycin for treatment of infection. There were 12 children who were treated according to the protocol and in 7 of those (55%) MRSA was successfully eradicated[91]. McFarlane et al. reported a three-step protocol used in paediatric patients. Of the 17 patients treated according to this protocol, 47% was successfully decolonized upon completion of the first step of the protocol and 70.5% upon completion of the second step. After the intravenous treatment, 94% of patients were cleared of MRSA[92]. Another study evaluated the use of rifampicin and sodIUm fusidate over a six-month treatment period, which was successful in 5 out of 7 patients[93]. Since the studies evaluating different protocols are small and are inhomogeneous with regard to follow-up time, it is not entirely clear which protocol is most appropriate. Drug-safety, the emergence of intermediate susceptibility of *S. aureus* or other pathogens to drugs used in combating MRSA, are issues that need to be taken into account upon choosing eradication protocols. Apart from the use of antibiotics to eradicate MRSA, the adherence to hygiene measures and the segregation of patients according to their MRSA status is recommended[94].

BURKHOLDERIA CEPACIA COMPLEX

Burkholderia bacteria are Gram-negatives and were first referred to as belonging to the Pseudomonas genus. Genotype studies later revealed that the genotypic characteristics of this species were different from Pseudomonas species and therefore they were reclassified as Burkholderia species[95]. The discovery of further genotypic differences among *B. cepacia* bacteria led to the identification of nine species now referred to as the *B. cepacia* complex[96] (which includes *B. cepacia* genomovars I and III, *B. multivorans*, *B. stabilis* and *B. vietnamiensis*).

The identification of *B. cepacia* complex isolates is performed using selective media, conventional biochemical analysis or commercial systems. With the use of commercial systems, *B. cepacia* complex organisms have been misidentified, e.g. as *S. maltophilia, Pseudomonas spp.* and *Alcaligenes spp*[97]. Media developed for selective isolation of *B. cepacia* complex from CF sputum include *B. cepacia* selective agar (BCSA)[98;99]. There is a general consensus that isolates identified through commercial test systems as belonging to the *B. cepacia* complex,

Table 4 Eradication protocols for MRSA

author	year	CF center	
Macfarlane et al.[91]	2007	paediatric	Step 1: – topical mupirocin to anterior nares twice daily (5 days) – sodium fusidate 50mg/kg (5 days) – rifampicin 20–40 mg/kg/day (5 days) – chlorhexidine for washing Step 2: repeat step 1 for 5 days Step 3: – teicoplanin i.v. 10–15 mg/kg/day 12h x three doses then – teicoplanin i.v. 10–15 mg/kg/day once daily for 9–13 days
Garske et al.[92]	2004	adult	Six months rifampicin (600 mg/day or 450 mg/day if weight <50 kg) and sodium fusidate (250–500 mg twice a day)
Solis et al.[90]	2003	paediatric	– treatment of MRSA carrier: • nasal carriage: 2% mucpirocin cream 4 times/day or 2% vancomycin cream 4 times/day • oropharyngeal carriage: 2% vancomycin paste (0.5 g) 4 times/day or 2% vancomycin gel (0.5g) 4 times/day or 5mg vancomycin lozenges 4 times/day • gastrointestinal carriage: 40 mg/kg/day oral vancomycin suspension in 4 doses • skin carriage: 4% chlorhexidine bath/shower on alternate days – treatment of colonization • tracheostomy: 2% vancomycin cream 2 times/day, change of foreign body • lower airways: nebulised vancomycin 4mg/kg/dose, 4 times/day diluted in saline (preceded by nebulised salbutamol to prevent bronchoconstriction)

should be further tested for growth on BCSA, presence of lysine and ornithine decarboxylase activity, oxidation of sucrose and adonitiol, presence of oxidase activity, haemolysis, pigment production and growth at 42°C.

EPIDEMIOLOGY

The emergence of *Bulkhorderia cepacia* was noted in the 1980's. Today the *B. capacia* complex is a well recognised pathogen in CF patients. The overall mean prevalence of *B. cepacia* was 2.8% in US patients[84]. In a study from a CF unit in the UK, the prevalence of *B. cepacia* increased before 1990, but decreased again until 4% in 2005[85] . The Utrecht CF center prevalence of *B. cepacia* is similarly low, as shown in Table 1.

An important aspect of *B. cepacia* is that transmission between patients has contributed to its prevalence. This transmission was found to occur not only in hospitals (there have been CF centers in which outbreaks with *B. cepacia* were reported[100]) but the spread of *B. cepacia* also occurred through intimate or frequent social contact between patients[101]. To limit the spread of *B. cepacia* infection control measures were put into practice in many CF centers. These included segregation of colonized patients, discouragement of social contact between infected patients and the discontinuation of summer camp sponsorship and support[102].

CLINICAL IMPACT

The first description of the clinical impact of *B. cepacia* (then still referred to as *P. cepacia*) in CF patients appeared in 1984. This report describes the 'cepacia syndrome', which was characterized by a severe deterioration of lung function with a bacteraemia[103]. Subsequently, several studies have consistently shown a more rapid lung function decline in patients after *B. cepacia* infection. In a case-control study, mortality 1 year after colonization was significantly higher when compared to mortality in controls, 1 year after the colonization date of the matched case. Also, lung function decline was more rapid in colonized patients, suggesting an independent effect of *B. cepacia*[104]. In analogy to these results, a study of children with CF following a hospital outbreak of *B. cepacia*, showed significantly greater lung function deterioration in children who became colonized with *B. cepacia*, compared with those who did not become colonized[100]. Moreover, in a large epidemiologic study on a 5-year survivorship model of CF, *B. cepacia* was the strongest predictor of 5-year survival, suggesting an independent effect of *B. cepacia* on morbidity[1].

MANAGEMENT

B. cepacia complex isolates are intrinsically resistant to aminoglycosides, and frequently are multiresistant. Therefore, eradication is often not successful, but therapy is often aimed at decreasing bacterial load during exacerbations. *B. cepacia* complex isolates may be susceptible to carbapenems, ceftazidime, quinolones and trimethoprim-sulfamethoxazole[105]. In a study on 119 multi-drug resistant *B. cepacia* isolates, multi combination bactericidal testing (MCBT) showed that triple antibiotic combinations containing tobramycin, meropenem and a third additional antibiotic were most effective[106]. These combinations were bactericidal against 81-93% of isolates. Although in vitro susceptibility testing can be used to guide the choice of therapeutic agents against *B. cepacia*, further studies are needed to investigate whether these test results are associated with clinical efficacy.

CONCLUDING REMARKS

Many pathogens play a role in CF lung disease, and it is conceivable that new pathogens will be added to the array of those already known to be present in CF. With the multifactorial nature of CF lung disease in general and the polymicrobial nature of CF lower airway disease in particular, it is difficult to assess the exact role of these organisms in CF lung disease progression. Prospective studies are needed to determine which organisms are merely colonizers of damaged lungs, and which are true pathogens contributing to the progression of lung disease.

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Chapter 3a

RESPIRATORY SYNCYTIAL VIRUS (RSV) INFECTION FACILITATES ACUTE PSEUDOMONAS AERUGINOSA COLONIZATION IN MICE

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JOURNAL OF MEDICAL VIROLOGY 2009 DEC;81(12):2096-103.

Abstract

Pseudomonas aeruginosa causes opportunistic infections in immunocompromised individuals and patients ventilated mechanically and is the major pathogen in patients with cystic fibrosis, in which it causes chronic infections. Epidemiological, in vitro and animal data suggest a role for respiratory virus infections in facilitating colonization and infection with P. aeruginosa. A study was undertaken to determine whether respiratory syncytial virus (RSV) infection could facilitate the initiation of an acute infection with P. aeruginosa in vivo. Balb/c mice were infected intranasally with P. aeruginosa, with and without simultaneous inoculation with RSV. Lung function measurements were undertaken using Whole Body Plethysmography and lungs were harvested 24 hours after inoculation. Mice exposed to RSV and P. aeruginosa showed 2000 times higher colony forming unit (CFU) counts of P. aeruginosa in the lung homogenates when compared to mice which were only infected with P. aeruginosa and lung function changes were more severe in co-infected mice. Control mice receiving RSV alone showed no significant changes in lung function or cytokine production, and no inflammatory changes in the lung parenchyma. These results suggest that RSV can facilitate the initiation of acute P. aeruginosa infection without the RSV infection being clinically apparent. This could have implications for treatment strategies to prevent opportunistic P. aeruginosa lung infection.

Introduction

Pseudomonas aeruginosa occurs ubiquitously throughout the environment but rarely causes disease in immunocompetent individuals, even though it is present in water and the gastrointestinal or upper respiratory tract[1]. In immunocompromised individuals or patients ventilated mechanically *P. aeruginosa* causes opportunistic infections like pneumonias, medical device-related infections and super infection of burn wounds[2]. *P. aeruginosa* is the principal infectious pathogen in cystic fibrosis, a hereditary disease characterized mainly by chronic infections of the respiratory tract. In these patients up to 80% develop a chronic infection which is accompanied by significant morbidity and eventually mortality[3].

Factors resulting in a predisposition to infection with *P. aeruginosa* are poorly understood. Decrease of innate or adaptive immunity in the immunocompromised host and decrease of local defense mechanisms in patients with burn wounds or mechanical ventilation might play a role. In patients with cystic fibrosis, dehydrated airway secretions and mucus plugging, altered composition of airway surface liquid and impaired host defense against *P. aeruginosa* are suggested[4]. Additionally, ceramide accumulation has been reported to facilitate *P. aeruginosa* infection in a cystic fibrosis mouse model[5]. Furthermore, several bacterial factors of *P. aeruginosa* are thought to contribute to the establishment and persistence of chronic infection, such as the ability to form biofilms, conversion to a mucoid phenotype and the accumulation of multiple mutations over time, including some which may promote persistence and chronic infection[6].

It has been suggested that viruses can play a role in the initiation or facilitation of *P. aeruginosa* respiratory infections in previously healthy subjects. In a 25 year retrospective study in Danish patients with cystic fibrosis, the onset of initial colonization and chronic infection with *P. aeruginosa* was observed to have a seasonal pattern that paralleled the respiratory virus season[7]. In another study on viral respiratory infections in cystic fibrosis, viral infections like respiratory syncytial virus (RSV) were shown to be more common in patients who developed a chronic infection with *P. aeruginosa* during the study period than in patients who did not develop a chronic infection, and these infections were frequently associated with a rise in *P. aeruginosa* antibodies in patients who were chronically infected with this pathogen[8].

Additionally, several in vitro studies have investigated the role of viruses in *P. aeruginosa* adherence to and infection of epithelial cells. In a study on adherence of bacteria to murine tracheas *P. aeruginosa* adhered to the desquamating cells of tracheas infected with influenza but not to normal mucosa[9], suggesting that the influenza virus might play a facilitating role in the pathogenesis of *P. aeruginosa* infection. In a mouse model of RSV and *Streptococcus pneumoniae*, the clearance of *S. pneumoniae* was found to be decreased following RSV infection[10]. Furthermore, in vitro studies in human airway epithelial cells showed an increase in adherence of *P. aeruginosa* to cells pre-infected with RSV. Even simultaneous infection of virus and bacteria resulted in enhanced adherence of *P. aeruginosa*[11].

The present study builds on previous epidemiological and in vitro observations and investigates whether simultaneous RSV infection can facilitate the initiation of an acute infection with *P. aeruginosa* in otherwise healthy mice. To this end an acute lung infection mouse model was used, with intranasal inoculation of the pathogens. The primary goal was to investigate the effect of RSV on the pulmonary bacterial load after acute infection with *P. aeruginosa*. Secondarily the functional effects were studied by measuring associated changes in lung function and the inflammatory response by evaluating lung histopathology and cytokine production in bronchoalveolar lavage fluid. The data reported here demonstrate that RSV infection can facilitate the initiation of an acute infection with *P. aeruginosa* in otherwise healthy mice.

MATERIALS AND METHODS

MICE

Female Balb/c mice aged 12-15 weeks were obtained from Taconic (Tornbjerg, Denmark). After transportation the mice were allowed to recover for at least one week, before being used in experiments. The mice were housed in cages of 5, in a clean experimental unit (specific pathogen-free) with barrier provisions and systematic health monitoring, at the Panum Institute, Copenhagen, Denmark. The mice received commercial food and water ad libitum. After inoculation with RSV, the mice were housed in an isolated cabinet. All cages were placed on a heat pad for several hours after infection (Scanbur, Karlslunde, Denmark). The local animal care committee approved the procedures performed on the mice in this study.

BACTERIA

A clinical *P. aeruginosa* isolate was obtained from a patient with cystic fibrosis who attended the cystic fibrosis center at the Rigshospitalet, Copenhagen, Denmark. It was the first isolate obtained in this patient, and has been described[12]. Bacteria were inoculated in Luria-Bertani (LB) medium and grown shaking overnight at 37°C. Serial dilutions were made with sterile 0.9 % saline, which were plated on blue agar plates (a modified Conradi Drigalski's medium selective for Gram-negative rods; State Serum Institute, Copenhagen, Denmark) for determination of colony-forming units (CFU) after overnight incubation at 37°C. The dilution factor that yielded around 1 x 10^6 CFU/ml was determined and used for diluting the overnight culture with sterile saline. Inocula were confirmed by plating the serial dilutions.

VIRUS

RSV serotype A was used in all experiments[13]. Stock aliquots contained 1.26 x 10^7 plaque-forming units (PFU)/ml and were stored at -80°C.

INTRANASAL INOCULATION

Mice were anaesthetized by intraperitoneal injection of a freshly prepared mixture of ketamine (65 mg/kg), xylazine (13 mg/kg) (both Intervet, Skovlunde, Denmark) and sterile isotonic saline. Mice were held in an upright position and while pushing their mandible to close the mouth, 50 µl of RSV inoculum (activity 0.63 x 10^6 PFU) was applied drop wise to the nostrils with a pipette, followed by 50 µl (~0.5 x 10^5 CFU) of bacterial inoculum. Mice were held upright until the inoculum was inhaled completely. A period of 60 minutes was allowed between the viral inoculation and the bacterial challenge, to prevent the mice from inhaling large amounts of fluid at once. Control mice received bacteria, RSV or sterile saline only.

VALIDATION OF THE NASAL INOCULATION MODEL

Nasal application of *P. aeruginosa* or RSV has been described previously[13;14]. In order to investigate whether this would be a practical method of administering a sufficient amount of inoculum to the lungs after nasal inoculation, a radio-labelled peptide ((125)I-TFF2) was used [15]. Mice were anaesthetized as described above. The mice were then held in an upright position and while pushing the mandible to close the mouth, 50 µl of the peptide solution was placed over the nostrils. Mice were held in an upright position until the solution was inhaled completely. The mice were then killed using pentobarbital and the lungs and stomach were removed. Radioactivity was measured in the

lungs, stomach and a control amount of 50 µl of the peptide solution using a gamma counter.

MEASUREMENT OF PULMONARY FUNCTION

To quantify changes in respiratory physiology induced by infection Whole Body Plethysmography (WBP) was used in unrestrained mice (Buxco, Troy, NY). Before each measurement, each chamber was calibrated to ambient pressure and temperature by rapidly introducing 1 ml of air into the chamber. Each mouse was placed inside a separate chamber and was provided with fresh air through a bias flow inlet. Readings were taken for 5 minutes, in which pressure differences between the main chamber (containing the mouse) and the reference chamber were measured. Several respiratory variables including enhanced pause (PenH; a non-dimensional measurement that correlates with airway resistance[16]) were calculated using software supplied by the manufacturer. Lung function was measured at baseline in all mice (15 mice per treatment group), and again 24 hours after inoculation, right before termination of the experiment.

LUNG BACTERIOLOGY EVALUATION

After completion of lung function measurements 24 hours after inoculation, mice were killed using intraperitoneal pentobarbital (200mg/ml)/lidocaine (20 mg/ml) injection (10 mice per group in each experiment). Lungs were removed and homogenized on ice at 13,500 rpm in 3 ml sterile saline, using a Heidolph diax 600 homogenizer (Struers, Denmark). The homogenates were serially diluted in sterile saline. 100 µl of the homogenate and 100 µl of every dilution were plated on blue agar plates and incubated overnight at 37°C for counting of colony-forming units (CFU). Colonies were assessed by appearance and development of a deep blue color within 10 seconds when stained with oxydase reagens (State Serum Institute, Copenhagen, Denmark) to detect *P. aeruginosa* (presence of the enzymecomplex cytochrome c).

BRONCHOALVEOLAR LAVAGE FLUID STUDIES AND HISTOPATHOLOGY

In each group 5 mice were randomly selected to undergo broncho alveolar lavage after having been killed as described above. The trachea was exposed and a venflon catheter (22G) was inserted into the trachea. Lavage was done by flushing the lungs once with 0.7 ml of sterile 0.9 % saline. Lavage fluid was immediately frozen on dry ice and later stored at -80°C. Antibody bead kit assays (mouse 5-plex and 10-plex) were performed to measure

cytokines (Biosource, Camarillo, CA). Concentrations were measured of pro-inflammatory cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, GM-CSF (which promotes granulocyte and monocyte production), IFN-γ, and TNF- α. Additionally 5 chemokines were included in the assay: KC (an IL-8 homologue), MCP-1, MIG (monokine induced by IFN-γ), IP10 and MIP-1α. Measurements were done using a Luminex 100 IS (Luminex Corporation). Starstation software (Applied Cytometry Systems, Sheffield, UK) was used for analysis and acquisition of data. For histopathology studies, lungs were fixed in situ by intratracheal injection of 4% buffered formaldehyde, removed and put into more fixative. After fixation the lungs were embedded in paraffin wax and cut into 5µm sections, followed by staining with hematoxylin and periodic-acid Schiff (PAS). The samples were evaluated blind by two pathologists.

STATISTICAL ANALYSIS

Non-Gaussian distributed results were compared using Mann Whitney U-test. Lung function measurements were compared by unpaired Student's t-tests. Level of significance: $p<0.05$. SPSS version 15 for Windows was used.

RESULTS

NASAL INOCULATION

Effectiveness of nasal application was investigated by administration of 50µl of radio-labelled peptide solution followed by excision of the lungs and stomach for radio-active particle counting. The counts detected in the lungs and stomachs of the mice were compared to an equivalent sample of the original peptide solution. The mean percentage of radioactive particles detected in the lungs (relative to the control fluid) was 71% (range 61-76%). The stomachs of the mice did not contain any detectable radio-active particles indicating that the fluid was delivered to the lungs without also being swallowed.

EFFECT OF SIMULTANEOUS RVS INFECTION

LUNG BACTERIAL LOAD

Mice were infected with RSV and *P. aeruginosa* simultaneously, or only with *P. aeruginosa* (approximately 0.5×10^5 CFU/mouse). Lungs were harvested 24 hours after infection and quantitative cultures of lung homogenates were performed. Mice exposed to both RSV and *P. aeruginosa* showed about 2000 times higher CFU counts of *P. aeruginosa* in the lung homogenates when compared to mice infected only with *P. aeruginosa* (median 3.05×10^1 CFU/ml of lung homogenate for mice infected with bacteria only and 6.0×10^4 CFU/ml of lung homogenate for co-infected mice ($p<0.01$) (Figure 1)).

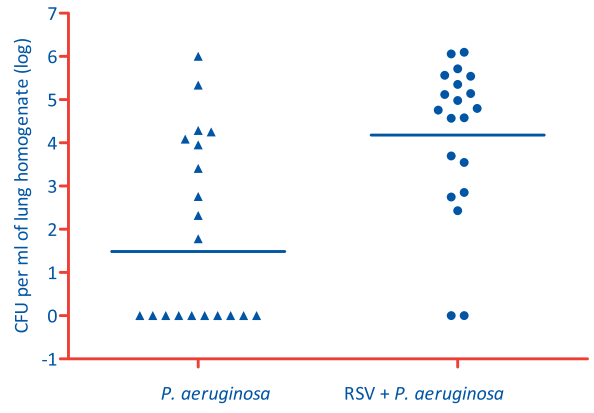

Figure 1 Bacteriology of lung homogenates (n=10 in each group, results of two experiments, each showing a significantly higher median number of CFU for co-infected mice), representing the number of CFU per ml of lung homogenate, 24 hours after inoculation with 0.5×10^5 CFU per mouse. Bars represent median number of CFU per group.

LUNG FUNCTION

To evaluate the respiratory physiology changes, lung function measurements were performed in unrestrained mice at baseline and 24 hours after inoculation. Measurements were also performed in control mice inoculated with only saline or RSV. Baseline values of Penh (enhanced pause, a measure of airway resistance), minute ventilation and breathing frequency were similar in all groups of mice (Figure 2). After 24 hours, co-infected mice showed a lower breathing frequency when compared to the mice infected only with bacteria (332/min vs. 381/min respectively, $p<0.05$). The breathing frequency in the RSV control mice was no different from the saline control group (mean 494/min vs. 464/min respectively, $p>0.05$). A similar effect was observed for minute ventilation. The co-infected mice had significantly lower minute ventilation when compared to the group infected only with *P. aeruginosa* (65.6 and 85.6 ml respectively, $p<0.05$). RSV control mice showed no significant difference compared to the saline group (103.6 ml and 95.7 ml respectively, $p>0.05$). Enhanced pause (Penh) was elevated in both groups of mice infected with bacteria (1.74 for co-infected mice and 1.37 for mice infected with *P. aeruginosa*, $p>0.05$) and was not different in the RSV group compared to the saline group (0.63 and 0.57, $p>0.05$).

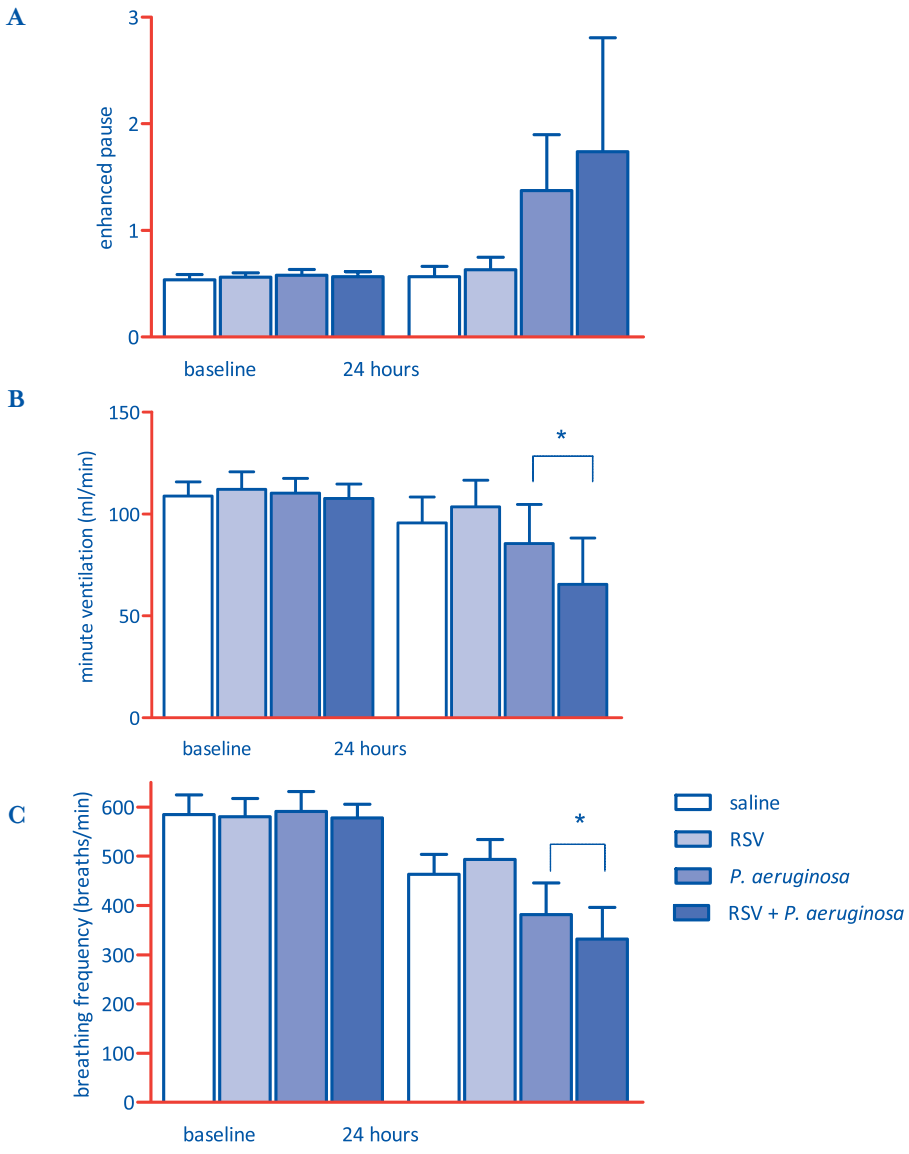

Figure 2 Enhanced pause (A), minute ventilation (B) and breathing frequency (C) measured at baseline and one day after simultaneous exposure to either RSV, *P. aeruginosa*, saline or both. Boxes represent mean values per group of mice (n=15 in each group), with bars showing SD. *$p<0.05$

BRONCHOALVEOLAR LAVAGE FLUID

Cytokine analysis of the lavage fluid was performed to evaluate the production of pro-inflammatory cytokines and chemokines in the lungs. IFN-γ was undetectable in all groups and IL-2, IL-4 and IL-5 gave unreliable standard curves, and therefore these results were omitted. For all cytokines, mice infected with RSV alone showed no significant production of cytokines when compared to control mice that received saline. Production of KC (IL-8 homologue) was observed but this was minimal (mean 22.3 pg/ml (SD 19.32) for RSV infected mice and 0.0 pg/ml (SD 0.0) for control mice, p=0.06). There was increased production of the measured cytokines in mice infected with *P. aeruginosa* only and co-infected mice. However, comparison of these two groups of mice showed no significant differences in cytokine production, except for a minimal difference in MIG (a T-cell chemoattractant) production (54.8 pg/ml (SD 30.3) for *P. aeruginosa* infected and 22.1 pg/ml (SD 8.5) for co-infected mice, p=0.03).

HISTOPATHOLOGY

Histopathology slides were evaluated and scored for severity of inflammatory changes of lung tissue and bronchial epithelial changes. Scoring was done on a 5-point scale, and the average was calculated for results from the two observers (Table 1). Examination of the lungs of mice infected with *P. aeruginosa* and with *P. aeruginosa* and RSV showed a similar severity of inflammation in the lung parenchyma. The inflammation was characterized by perivascular and alveolar infiltration with inflammatory cells (Figure 3 c and d). The lungs of control mice receiving saline or RSV did not show substantial inflammatory changes in the lung parenchyma. Epithelial damage was observed in all groups of mice to some degree, but was most severe in the mice infected with RSV only. Bronchial epithelial cells of these mice showed a high degree of vacuolization and sloughing of nonciliated epithelial cells (Clara cells) and also damage to ciliated epithelial cells. This was more pronounced distally i.e. in the bronchioles (Figure 3b).

Table 1 Histopathology scores of mouse lungs (n=5 mice in each group)

	saline	RSV	*P. aeruginosa*	RSV + *P. aeruginosa*
epithelial damage	-/+	+++	++	+
inflammation	-/+	-/+	++	++

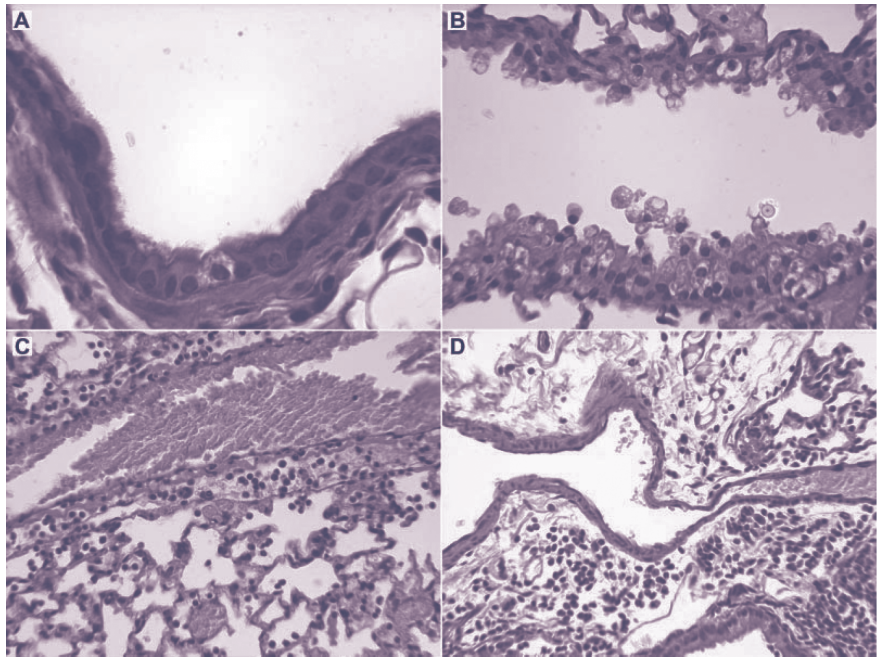

Figure 3 Histopathology of mouse lungs 24 hours after inoculation with saline (A), RSV (B), *P. aeruginosa* (C) or RSV and *P. aeruginosa* (D). RSV-infected control mice display extensive damage to cells of the bronchial epithelium. Mice infected with *P. aeruginosa* alone or RSV and *P. aeruginosa* show inflammatory changes in the lung parenchyma. Magnification 320 x (c,d), 500 x (b) and 800 x (a).

DISCUSSION

Findings from in vitro experiments suggest that viral infections can have a facilitating role in *P. aeruginosa* airway infection[11]. Clinical observations provide circumstantial evidence for this enhancing effect in cystic fibrosis[7;8]. The aim of the experiments presented here was to study the in vitro findings in an in vivo model. Lung bacterial load and associated lung function changes were compared between mice infected with RSV and *P. aeruginosa* and mice infected only with *P. aeruginosa*. This showed that co-infected mice had a higher bacterial load and more severe lung function changes, suggesting that *P. aeruginosa* was better able to establish an infection in the presence of an RSV infection. Evaluation of the associated immunological response by histopathology and cytokine analysis showed inflammatory changes in both groups of mice infected

with bacteria, but no parenchymal inflammation in control mice infected only with RSV. This suggests that in this early infection model the facilitating effect of RSV occurred independent of an immunological response to the virus.

A facilitating role in bacterial infections by viruses has been reported widely for various pathogenic agents[17]. Several mechanisms are suggested that could explain an enhancing effect of viral infections like RSV on acute pulmonary infection with *P. aeruginosa*. The first is that respiratory viral infections disrupt normal tissue architecture of the respiratory epithelium and cause loss of cilia, which might allow bacteria to adhere more easily[18]. This effect has been observed in a mouse model of influenza, where *P. aeruginosa* was able to adhere only to the desquamating cells of influenza-infected tracheas[9]. The second is that immunological changes caused by viruses can lead to less efficient clearance of bacteria. In a chinchilla model, influenza infection has been observed to reduce neutrophil functions including respiratory burst and bacterial killing[19]. In another study, clearance of *S. pneumoniae* after RSV infection in mice was significantly reduced 1-7 days following RSV exposure[10]. This effect of RSV on bacterial clearance was not specific to *S. pneumoniae*: similar decreases in clearance were observed for *Staphylococcus aureus*. The effect of RSV infection on *P. aeruginosa* (PA01) clearance after RSV infection was studied and in common with the present observations significantly higher colony counts were found in mice with RSV infection. These results were obtained after bacterial challenge 7 days following RSV exposure as opposed to the present study in which the bacterial challenge was performed simultaneously with viral inoculation. In the *S. pneumoniae* experiments, analysis of bronchoalveolar lavage fluid demonstrated a greater rise in macrophages and polymorphonuclear neutrophils in RSV-infected mice with *S. pneumoniae* when compared to controls. Neutrophil cellular myeloperoxidase levels were decreased in RSV infected mice which is suggestive of an alteration in neutrophil function being the possible cause of decreased bacterial clearance[10]. In another study, viral infection of mice with Lymphocytic Choriomeningitis Virus (LCMV), through induction of type I IFN, led to apoptosis of bone-marrow granulocytes and impaired granulocyte migration to sites of bacterial infection, suggesting that increased susceptibility to bacterial superinfection in viral disease may occur as a consequence of the innate antiviral response[20]. The third mechanism is increased adherence of pathogenic bacteria as a result of virus-induced upregulation of cellular molecules that act as receptors for pathogenic bacteria. Bacteria can bind to virus-induced glycoproteins expressed on the surface of infected cells, e.g. glycoprotein G and F[17]. Additionally, in vitro studies showed direct binding

between RSV and *S. pneumoniae* and RSV and *P. aeruginosa*, suggesting that RSV acts as a coupling agent between these bacteria and respiratory cells[11;21].

The exact mechanisms responsible for the enhancing effect of RSV on *P. aeruginosa* airway infection in the studies presented require further study. Virus-induced changes in the immunologic response may have a role. However, control mice receiving only RSV did not show significant changes in lung function and minimal upregulation (although not significant) of cytokine production. Histopathological evaluation of the lungs showed damage to the airway epithelium but no alveolar tissue inflammation. This suggests that the inflammatory response to RSV is not substantial 24 hours after inoculation. This is in accordance with studies of primary RSV infections in Balb/c mice, where viral titres peak after 4-6 days, around which time pathological changes are observed in lung tissue[22;23]. Others have observed that RSV induced profound effects on ciliated cells of the respiratory epithelium, including loss of cilia and sloughing of cells[24]. In the present model damage to the respiratory epithelium was also observed, especially in non-ciliated epithelial cells (Clara cells). Virus-induced damage to airway epithelium could have been a contributing factor to the facilitating effect of RSV. Promotion of bacterial adhesion to the respiratory epithelium by viruses has also been observed for other viruses and bacteria[25]. These findings are important since adherence of bacteria has been shown to be an important first step in the establishment of bacterial pneumonia in a mouse model[26]. Additionally, virus-induced loss of ciliary function could have impaired the mucociliary clearance mechanism, leading to an inability of virus-infected mice to successfully clear the bacteria. Furthermore, since the virus and bacteria were added simultaneously, it is a possibility that direct binding between *P. aeruginosa* and RSV, as observed in vitro[11], played a role in the study presented here. Hament et al. demonstrated a similar effect, when studying the effect of RSV on *S. pneumoniae* infection[13]. In their in vitro studies, RSV was shown to increase adherence of pneumococci and they also observed direct binding between the virus and the bacteria. In a mouse model, they observed an enhancing effect on bacteraemia in the presence of RSV, which was strongest when RSV and *S. pneumoniae* were added simultaneously. Similarly, in a mouse model of pneumococci and influenza virus, the virus increased mortality in pneumococcal infection in mice, and an additional effect in mortality rates was observed when mice were simultaneously inoculated with virus and bacteria[27].

The clinical importance of the present findings is that they provide some insight into the role of respiratory viral infections in *P. aeruginosa* airway infections. The results confirm observations from in vitro experiments. Furthermore, these data support circumstantial clinical evidence for a facilitating role in colonization with *P. aeruginosa* as observed in patients with cystic fibrosis. In contrast to other studies on virus-bacterial interaction, an early infection model was used, in which the RSV infection did not last beyond 24 hours, showing that the facilitating effect of RSV occurs within the initial phase of infection, without the presence of a clinical effect of RSV or a substantial inflammatory response. This could have implications for possible strategies to prevent the opportunistic colonization with *P. aeruginosa*, suggesting that the enhancing effect can take place during simultaneous infection. For this purpose, prevention of respiratory viruses by immunization or prophylactic use of antibiotics during the respiratory virus season could be possible treatment options, for instance in patients with cystic fibrosis. For RSV no vaccine is available at present[28], but the use of passive prophylaxis with palivizumab has been suggested to be of possible benefit in reducing hospitalizations in a recent retrospective study in young CF patients[29]. Further prospective studies are needed to investigate the effect of RSV infection prevention on reduction of bacterial infections.

In conclusion the present study shows that in a mouse model, acute airway colonization with *P. aeruginosa* can be facilitated by viral infections like RSV: The presence of an RSV infection led to increased lung bacterial loads, and more severe lung function changes. This effect occurred during simultaneous infection with RSV, without the occurrence of a significant immune response, suggesting that even without the virus being clinically apparent, its facilitating effect can take place. This could be important in the understanding and prevention of opportunistic airway infection with *P. aeruginosa*.

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Chapter 3b

THE EFFECT OF HEPARIN ON RESPIRATORY SYNCYTIAL VIRUS (RSV) – PSEUDOMONAS AERUGINOSA CO-INFECTION IN MICE

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Abstract

Background

Several studies showed a facilitating role for respiratory syncytial virus (RSV) in facilitating Pseudomonas aeruginosa infection. In vitro studies suggested that a direct binding between the virus and bacteria in which the viral glycoprotein G might be responsible for the RSV — P. aeruginosa interaction. Enhancement by RSV was abrogated upon treatment of the cells with heparin, which also binds glycoprotein G, thereby blocking the site for P. aeruginosa — RSV binding. We aimed to study the effect of heparin on the influence of RSV-infection in an acute P. aeruginosa mouse model. We hypothesized that heparin treatment abrogates the enhancing effect of RSV on P. aeruginosa lung infection.

Methods

Balb/c mice were inoculated with RSV. Twenty-four hours later, heparin or saline were administered, followed immediately by P. aeruginosa. Lung function was measured at baseline and before termination of the experiment using whole body plethysmography (WBP). Symptom severity score was recorded after P. aeruginosa infection. Mice were sacrificed 48 hours after start of the experiment. Lung bacteriology and histopathology were performed.

Results

There were no statistically significant differences in the median numbers of CFU between the treatment group and control mice (p=0.49). Mice treated with heparin had a significantly lower breathing frequency 48 hours after start of the experiment than control mice (301 breaths/min (SD 8.50) and 345 breaths/min (SD 12.11) respectively; p=0.007). There were no differences in lung histopathology inflammation score.

Conclusion

In conclusion, the results presented here suggest that in this mouse model of RSV and P. aeruginosa, intranasal therapy with heparin did not result in a less severe pulmonary infection. The decreased lung function in heparin treated mice might suggest heparin is unsafe for use in this setting.

Introduction

Several epidemiology studies have suggested a role for respiratory viruses like respiratory syncytial virus (RSV) in establishing an airway infection with *Pseudomonas aeruginosa* in cystic fibrosis patients[1-3]. In vitro studies investigating this possible interaction of *P. aeruginosa* and RSV suggested that a direct binding between the virus and bacteria might contribute to an enhancing effect of RSV on *P. aeruginosa* infection[4]. In a mouse model, we previously showed that simultaneous infection with RSV and *P. aeruginosa* can facilitate acute *P. aeruginosa* colonization[5]. The in vitro studies further suggested that the viral glycoprotein G might be responsible for the RSV – *P. aeruginosa* interaction, as *P. aeruginosa* could bind to this glycoprotein. This was supported by the observation that the enhancement by RSV was abrogated upon treatment of the cells with heparin[4], which also binds glycoprotein G, thereby blocking the site for *P. aeruginosa* – RSV binding. A similar effect was found in a mouse model of RSV and pneumococci, in which the enhancing effect of RSV was lost when mice were treated with heparin[6;7]. This possible preventive effect of heparin could have important implications in CF patients. Therefore we aimed to study the effect of heparin on the influence of RSV-infection in an acute *P. aeruginosa* mouse model. We hypothesized that heparin treatment abrogates the enhancing effect of RSV on *P. aeruginosa* lung infection.

METHODS

ANIMALS

Female Balb/c mice aged 12–15 weeks (Taconic, Tornbjerg, Denmark) were used (see graphs for number of mice used per experiment). After transportation the mice were allowed to recover for at least 1 week, before being used in experiments. The mice were housed in filtertop cages of four, in a clean experimental unit (specific pathogen-free) with barrier provisions and systematic health monitoring, at the Panum Institute, Copenhagen, Denmark. The mice received commercial food and water ad libitum. Heat pads were used to warm the cages of the mice to 24°C after inoculations, as the mice were immobilized for approximately 30 minutes. The local animal care committee approved the procedures performed on the mice in this study.

BACTERIA

A wild-type strain of *P. aeruginosa* was used (PA01, Iglewski variant)[8]. Bacteria were inoculated onto blue agar plates (modified Conradi drigalski's medium, State Serum Institute, Copenhagen, Denmark) from a frozen stock, and grown overnight at 37°. A small inoculating loop of colonies were selected and suspended in 50 ml LB broth for growing overnight, shaking at 37°. A hundred µl of the overnight culture was centrifuged. The cells were washed twice with sterile saline, and resuspended in 5 ml of NaCl, yielding a 50x dilution. The inoculum strength was approximately 1.8 x 10^8 CFU/ml.

VIRUS

RSV serotype A was used as described before[5]. The activity was approximately 1,3 x 10^7 PFU/ml. The virus was stored in -180° (in liquid nitrogen) and was thawed shortly before inoculating the mice.

HEPARIN

Heparin was used in a dose of 0.5 IU per mouse (as determined from pilot experiments, since higher doses gave macroscopic hemorrhages in mouse lungs). Heparin solution (leopharma, Denmark) with a concentration of 500 IU/ml was used, and was diluted for experiments using sterile saline.

INFECTION MODEL

Mice were anaesthetized by intraperitoneal injection of a freshly prepared mixture of ketamine hydrochloride (65 mg/kg) and xylazine (13 mg/kg) (both Intervet, Skovlunde, Denmark). With mice held in an upright position, the inoculum was instilled dropwise to the nares. First RSV was administered. Twenty-four hours later, heparin or saline were administered, followed immediately by *P. aeruginosa*. Heparin was administered in a volume of 25 µl. *P. aeruginosa* and RSV were administered in volumes of 50 µl. After inoculation, a heating mat was placed beneath the filtertop cages to keep mice warm until they had become fully mobilized. Mice were sacrificed 48 hours after start of the experiment by intraperitoneal injection of a lidocaine/pentobarbital mixture.

LUNG BACTERIOLOGY

Quantitative bacteriology was performed on randomly selected mice. After aseptic en-bloc removal from the thoracic cavity, lungs were homogenized in 3 ml of sterile saline, on ice. Of each homogenate, 100 µl was plated and serial dilutions were made for plating. The plates were incubated at 37° for approximately 20 hours and the plates were inspected for presence of *P. aeruginosa* colonies, as determined by using oxydase reagens.

LUNG FUNCTION AND BODY WEIGHT MEASUREMENTS

Lung function was measured using Whole Body Plethysmograpy (WBP) (Buxco, Troy, NY), which is a non-invasive method for evaluating lung function. Mice were put into separate chambers and readings were taken for 5 minutes, after giving the mice 1 minute to adjust to their new environment. WBP was performed at baseline and right before ending the experiment.

LUNG HISTOPATHOLOGY

After anesthesia, the lungs of a random selection of mice were prepared for histopathology studies. The trachea was exposed and a Venflon catheter (22 G) was inserted into the trachea. The lungs were slowly inflated with approximately 0.8 ml of 4% formaldehyde buffer. After several minutes, lungs were removed from the thoracic cavity and were left to fixate in formalin buffer for at least 7 days. Subsequently, the lungs were embedded in paraffin wax and cut into 5µm sections. The sections were mounted and stained with hematoxylin and periodic acid Schiff (to stain for exopolysaccharides). Lungs were scored for inflammatory changes and damage to bronchiolar epithelium by a pathologist who was blinded for the treatment which the mice received. Scoring was done on a 5-point scale, where 0 represents no inflammation and 5 represents severe inflammation.

SYMPTOM SEVERITY SCORE

Animals were scored on symptoms approximately 8 hours after *P. aeruginosa* challenge, by a researcher who was blinded to the treatment which the mice had received. Cumulative scores range from 0 to 13 and are adapted from Murphy et al[9]. Scores are based on the varying degree of symptoms of sickness, such as lesions, ruffled fur and neurological dysfunctions. Table 1 displays the scoring system.

STATISTICAL ANALYSIS

Non-normally distributed results were analyzed with the Mann-Whitney test and normally distributed results were analyzed with the Student's t-test. SPSS version 15 for Windows was used.

Table 1 Symptom severity score adapted from Murphy et al[9].

Eyes	0 1 2	— normal — infection one eye — infection both eyes
Fur	0 1	— well groomed — ruffled fur
Temperature	0 1 1	— warm — cold — shaking
Body position	0 1	— normal — hunched back position
Activity	0 1 2 3	— normal spontaneous activity — lower spontaneous activity — no sponteneous activity, but moving when touching mice — unresponsiveness
Grip strength	0 1 2	— normal — reduced — absent
Respiratory distress	0 1 1	— breathing normally — gasping — retractions
Urination and defecation	0 1	— clean mice — not washed
Touch escape	0 1 1	— normal — hypoactivity — aggressive

RESULTS

LUNG BACTERIOLOGY

Mice were anesthetized and received 50 µl of RSV intranasally. After 24 hours, mice were administered 25 µl of heparin (0.5 IU) or NaCl followed by 50 µl of *P. aeruginosa* (approximately 1 x 10^7 CFU per mouse). Mice were sacrificed 24 hours after *P. aeruginosa* challenge and lungs were aseptically removed in mice randomly selected for bacteriological culture/ evaluation. There were no statistically significant differences in the median numbers of CFU between the treatment group and control mice (p=0.49)(Figure 1). There were two mice in the heparin group that died after inoculation with heparin and *P. aeruginosa* when they were still anesthetized.

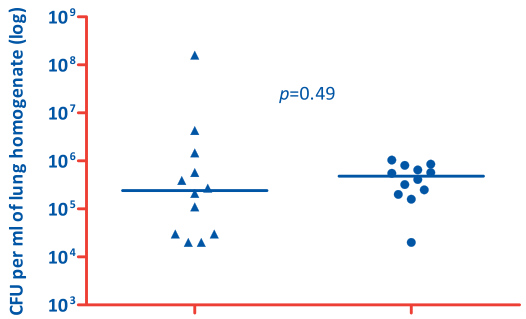

Figure 1 Bacteriology of mouse lung homogenates (bars represent median) (n=12 in control and heparin group)

LUNG FUNCTION

Baseline breathing frequency was not different between mice in the heparin group and those in the control group (603 breaths/min (SD 11.05) and 600 breaths/min (SD 10.41) respectively; p=0.86). Mice treated with heparin had a significantly lower breathing frequency 48 hours after start of the experiment (i.e. 24 hours after *P. aeruginosa* challenge) than control mice (301 breaths/min (SD 8.50) and 345 breaths/min (SD 12.11) respectively; p=0.007) (Figure 2). Minute ventilation and enhanced pause (penh) show similar patterns of no group difference for baseline values, but a statistically significant difference at 48 hours: heparin treated mice show a lower minute ventilation compared to control mice (54.47 (SD 2.63) ml/min and 75.07 (SD 4.98) ml/min respectively; p=0.001) and a higher Penh than control mice (2.91 (SD 0.24) and 2.15 (SD 0.22) respectively; p=0.03).

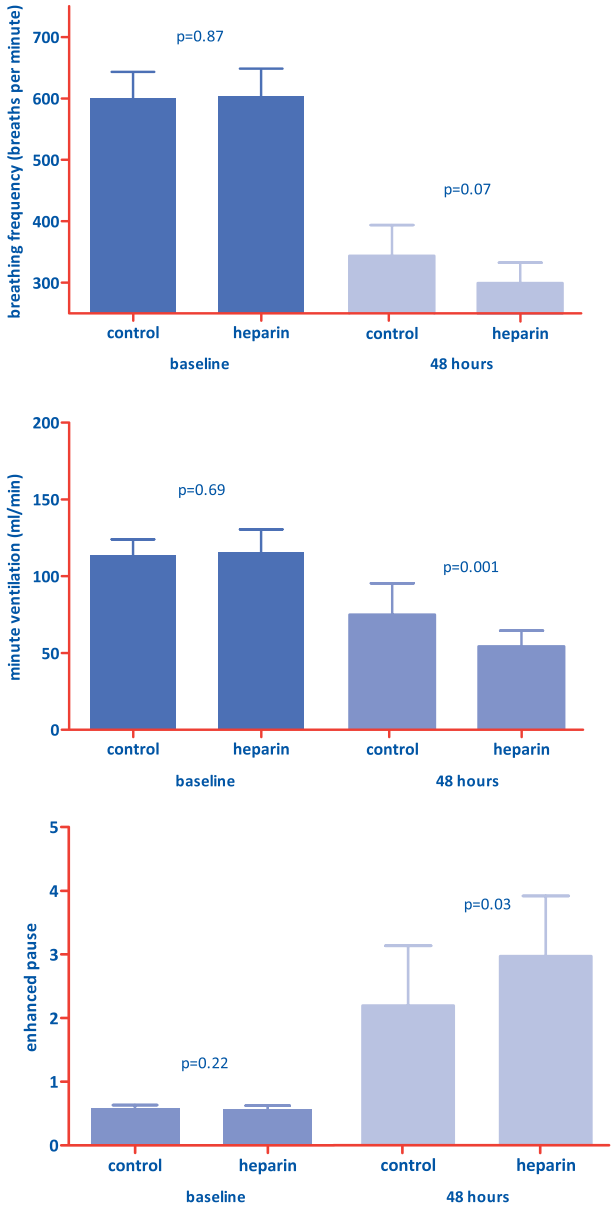

Figure 2 Lung function measurements at baseline and 48 hours after start of the experiment (bars represent mean, error bars represent SD) (n=17 mice per group).

LUNG HISTOPATHOLOGY

The lungs of mice in both groups showed inflammatory changes. However, there were no differences in inflammation score between mice treated with heparin and control mice (median 3 for both groups) (Figure 3).

SYMPTOM SEVERITY SCORE

All mice had symptoms of sickness, with a symptom severity score ranging from 5 to 7. Figure 4 shows the symptom severity scores for heparin treated mice and control mice. Although the median severity score of control mice (7) was higher than that of the heparin treated mice (6), this difference was not statistically significant (p=0.41).

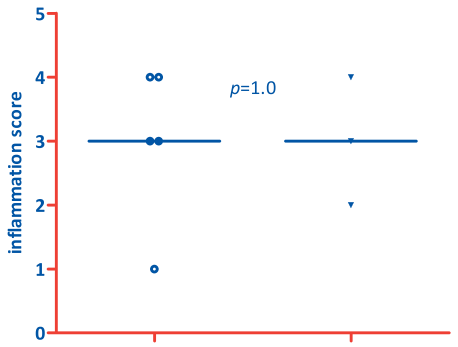

Figure 3 Inflammation score of lung histology sections for the control and treatment group (bars represent median) (n=3 for heparin group and n=5 for control group).

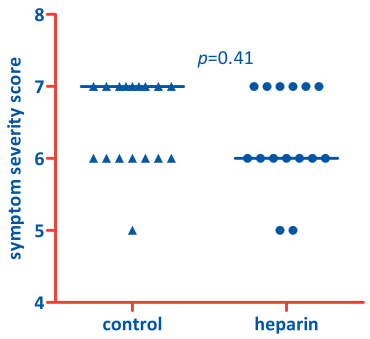

Figure 4 Symptom severity score 48 hours after start of the experiment for heparin treated mice and control group (bars represent median) (n=17 in control group, n=15 in heparin group).

DISCUSSION AND CONCLUSION

In the current study, we aimed to study the effect of intranasal heparin on RSV- *P. aeruginosa* synergism, by co-infecting mice with RSV and *P. aeruginosa* and treating with heparin. We hypothesized that heparin would abrogate the facilitating effect of RSV on *P. aeruginosa* infection. However, there was no significant difference in lung bacteriology of mice treated with heparin and control mice. The secondary endpoints of the studies presented here show that heparin treated mice had more severe deterioration of lung function 24 hours after *P. aeruginosa* challenge, although the symptom severity score was not significantly different.

The results here differ from previous studies. In vitro experiments investigating the effect of RSV on *P. aeruginosa* provided a basis for the here presented experiments. Van Ewijk et al. showed that simultaneous infection of RSV and *P. aeruginosa* increased adherence of *P. aeruginosa* to airway epithelial cells. Furthermore, upon pre-treatment of the cells with heparin, the enhanced adherence of co-incubated *P. aeruginosa* and RSV was nearly completely blocked[4]. In mouse studies by Hament et al. the enhancing effect of RSV on *S. pneumoniae* bacteremia was investigated, using a nasal inoculation model. These studies showed that simultaneous RSV and *S. pneumoniae* infection facilitates bacteremia and that after treatment with heparin, this facilitating effect of RSV was abrogated[7]. In the present study however, heparin treated mice had similar lung CFU levels, but significantly worse lung function compared to control mice, which could suggest an adverse effect of heparin. As pilot studies showed that the administration of higher doses of heparin (1 IU, 2,5 IU and 5 IU) were associated with (severe) macroscopic hemorrhaging of mouse lungs, the use of heparin in this mouse model seems unsafe.

There are several differences between the current experiments and the experiments by Hament et al. that could possibly explain why the effect of heparin was different. Firstly, Hament et al. investigated bacteremia development in mice intranasally infected with *S. pneumoniae*[7] whereas the experiments presented here use a model with lung infection as primary endpoint. It is possible that dissimilarities between the pathogenesis of *S. pneumoniae* bacteremia and *P. aeruginosa* lung infection are responsible for differences in the effect of heparin on the facilitating effect of RSV. Furthermore, heparin treatment in studies by Hament was simultaneous with RSV and bacterial challenge, whereas in the current experiment mice were infected with RSV 24 hours prior to

heparin and *P. aeruginosa* treatment. The reason for the chosen timing was that we aimed to study whether heparin would abrogate the direct binding between *P. aeruginosa* and RSV, as was observed in in vitro experiments by Van Ewijk et al[4]. If heparin and RSV were administered either simultaneously or if mice were pre-treated with heparin, any effect on the synergism between RSV and *P. aeruginosa* could be influenced by a possible effect of heparin on the infectiveness of RSV[10]. Therefore, the applied scheme of RSV pre-infection, followed 24 hours later by heparin and *P. aeruginosa* was chosen.

In conclusion, the results presented here suggest that in this mouse model of RSV and *P. aeruginosa*, intranasal therapy with heparin did not result in a less severe pulmonary infection. The decreased lung function in heparin treated mice might suggest heparin is unsafe for use in this setting.

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Chapter 4

ASPERGILLUS FUMIGATUS COLONIZATION IN CYSTIC FIBROSIS: IMPLICATIONS FOR LUNG FUNCTION?

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CLINICAL MICROBIOLOGY AND INFECTION 2011 SEP;17(9):1381-6.

Abstract

Background

Aspergillus fumigatus is commonly found in the respiratory secretions of patients with cystic fibrosis (CF). Although allergic bronchopulmonary aspergillosis (ABPA) is associated with deterioration of lung function, the effects of A. fumigatus colonization on lung function in the absence of ABPA are not clear.

Methods

This study was performed in 259 adults and children with CF, without ABPA. A. fumigatus colonization was defined as positivity of >50% of respiratory cultures in a given year. A cross-sectional analysis was performed to study clinical characteristics associated with A. fumigatus colonization. A retrospective cohort analysis was performed to study the effect of A. fumigatus colonization on lung function observed between 2002 and 2007. Longitudinal data were analyzed using a linear mixed model.

Results

Sixty-one out of 259 patients were at least intermittently colonized with A. fumigatus. An association was found between A. fumigatus colonization and increased age and use of inhaled antibiotics. In the longitudinal analysis, 163 patients were grouped according to duration of colonization. After adjusting for confounders, there was no significant difference in lung function between patients colonized for 0 or 1 year and patients with 2–3 or more than 3 years of colonization ($p=0.40$ and 0.64) throughout the study. There was no significant difference in lung function decline between groups.

Conclusions

Although colonization with A.fumigatus is more commonly found in patients with more severe lung disease and increased treatment burden, it is not independently associated with lower lung function or more severe lung function decline over a 5-year period.

Introduction

Cystic fibrosis is characterized by chronic infection of the airways in which *Pseudomonas aeruginosa* is the most important pathogen. Chronic infection with *P. aeruginosa* is associated with increased morbidity and mortality[1]. Filamentous fungi like *Aspergillus fumigatus* are also commonly found in respiratory secretions of patients. Reported prevalence rates of colonization in patients with cystic fibrosis vary, ranging from 6-58%[2-11]. The variation in the reported prevalence has been hypothesized to be attributable to age of patients and climatic conditions[8]. Colonization with *A. fumigatus* is defined as the presence of *A. fumigatus* in respiratory secretions and is different from the main clinical manifestation of *A. fumigatus* in CF, allergic bronchopulmonary aspergillosis (ABPA). This condition is characterized by pulmonary infiltrates and production of IgG and IgE antibodies specific for *A. fumigatus*, as well as a rise in total IgE[2]. The prevalence of ABPA varies between 2 and 8% and lung function has been shown to deteriorate over time in relation to *A. fumigatus* sensitization and ABPA[12].

The effect of colonization with *A. fumigatus* on lung function in the absence of ABPA is not clear. Case-reports of patients colonized with *A. fumigatus* whose respiratory deterioration did not respond to antibiotics, but was brought to a halt by antifungal therapy, suggest a direct negative effect of *A. fumigatus* on pulmonary condition[13]. Recently a longitudinal study has shown that chronic infection with *A. fumigatus* is associated with increased hospitalization[14]. However, in several cross-sectional studies there was no independent relationship between colonization and lung disease[6;11].

We aimed to study whether colonization with *A. fumigatus* was associated with unfavorable clinical outcome, by evaluating factors independently associated with *A. fumigatus* colonization and by longitudinally studying the effect of the duration of *A. fumigatus* colonization on the course of lung function.

METHODS

PATIENT SELECTION

The CF center in Utrecht currently provides care for around 350 patients. Patients undergo a routine yearly multidisciplinary examination, of which all results are prospectively recorded in a database. Demographics,

CF genotype, BMI (Body Mass index) values and pulmonary function data were collected from the database and were recorded during the yearly routine examination, which is considered as a moment of stability in patients' clinical condition. BMI Z-scores were calculated based on growth curves[15].

Patients were selected for two separate analyses. The first was a cross-sectional analysis of data from 2007 to study which factors were associated with *A. fumigatus* colonization, in order to generate a hypothesis on the effect of *A. fumigatus* colonization in our patient population. In this analysis, all patients who were under treatment at the center and who had been seen for routine care in 2007 were included. Patients were excluded if they were chronically infected with *B. cepacia* complex, if they had a history of lung transplantation or ABPA (defined according to diagnostic criteria[16]) and if they did not have pulmonary function or microbiology data available for 2007. The second analysis aimed to investigate the longitudinal effect of *A. fumigatus* colonization on lung function, to further study the hypothesis generated by the cross-sectional analysis; a retrospective cohort analysis was performed, in which all patients who were regularly followed at the CF center from 2002 to 2007 (a minimum of 4 years of lung function measurements and microbiology data) and who had a lung function measurement performed in 2002 (to ensure a baseline lung function value) were included. All patients gave written informed consent to store and evaluate their clinical data in the CF-database for scientific purposes and the use of this database is permitted by the ethical board.

MICROBIOLOGY

Sputum or throat swab samples were taken at each visit at the CF center from all patients. *A. fumigatus* and *P. aeruginosa* colonization status were investigated by evaluating cultures performed between 2002 and 2007. All samples were cultured according to standard diagnostic laboratory protocol, which included testing for fungi. Colonization with *A. fumigatus* in a given calendar year was defined as the presence of *A. fumigatus* in >50% of respiratory cultures performed in that year. Patients were categorized according to the number of years during the observation period in which they met the criteria for *A. fumigatus* colonization.

STATISTICAL ANALYSIS

For the cross-sectional data, simple logistic regression was performed to evaluate the association between the study variables and the dependent variable (*A. fumigatus* colonization). In order to study which variables were independently

associated with the outcome variable, variables showing an association (p<0.10) were entered into a multiple logistic regression model, using the 'enter' method. The number of variables in the model was limited to ensure at least 10 outcome events per independent variable[17].

Patients were divided into categories according to the number of years during the observation period in which they met the criteria for *A. fumigatus* colonization (group 1 no colonization- one year, group 2 two-three years and group 3 four years or more).

Differences among the three groups during the first year of observation were evaluated by the Kruskal-Wallis test (for non-normally distributed continuous variables), ANOVA (for normally distributed continuous variables) or the chi-squared test (for dichotomous variables). A linear mixed model was used to evaluate the effect of *A. fumigatus* colonization on lung function during the 5-year study period. The model assumed a linear trend in FEV_1% of predicted over time for each patient and allowed for random patient-specific slope and intercept. First, a basic model was created, with *A. fumigatus* colonization group, and random slope for time. To this model several possible confounders (variables associated with colonization in the cross-sectional analysis or those different for the three groups at baseline) were added, to check whether this significantly improved the model fit.

A difference in decline of FEV_1% of predicted among the three groups was tested by examining improvement in model fit after adding an interaction term to the model (time x group). This interaction term would allow for different slopes over time for the three groups. SPSS version 15.0 for Windows was used for all statistical analyses.

RESULTS

CROSS-SECTIONAL ANALYSIS

In 2007, 335 patients visited the CF center for annual check-up. Of these patients, 7 were excluded because they had a history of lung transplantation and 5 were excluded because they were chronically infected with *B. cepacia* complex. Nine patients did not have respiratory cultures taken at the CF center, 24 did not have lung function data available for 2007 (20 were too young for pulmonary function testing and 4 did not have pulmonary function test data available), and 31 were excluded because of a history of ABPA. In total, 259 were available for analysis.

Simple logistic regression indicated a significant association between the presence of *A. fumigatus* colonization in 2007 and increased age, decreased FEV_1 % predicted, chronic *P. aeruginosa* infection, increased number of hospitalizations and increased use of inhaled antibiotics and recombinant human DNase (rhDNase) (Table 1). In total, six variables with a p-value of <0.10 in the simple logistic regression were entered into the multiple logistic regression model: age, FEV_1, number of hospitalizations, inhaled antibiotics use, rhDNase use and chronic *P. aeruginosa* infection. In the final model *A. fumigatus* colonization was independently associated with increased age (p=0.02 and p= 0.09 for age categories 13-24 and ≥25 years respectively, versus reference category 0-12 years) and the use of inhaled antibiotics (p=0.001).

LONGITUDINAL ANALYSIS

Between 2002 and 2007, there were 225 patients who regularly visited the CF center (at least 4 years of lung function and microbiology data available). Six patients were excluded because of a history of lung transplantation and thirty patients were excluded because they had (developed) ABPA. Four patients were excluded because of chronic *B. cepacia* complex infection. Twenty-two patients did not have lung function data for 2002 available (20 because they were too young for spirometry and 2 because they did not have their lung function measured at this CF center). In total 163 patients were included in the longitudinal analysis.

There were differences in possible confounding clinical and demographic characteristics between the three groups: patients with longer duration of colonization were older (p<0.001), were more likely to use inhaled antibiotics (p<0.001), had more hospitalizations (p=0.02), and lower FEV_1 (p<0.001) and BMI z-score (p=0.04) (Table 2).

Table 1 Simple and multiple logistic regression analysis showing an independent association between *A. fumigatus* colonization and age and inhaled antibiotics use (n = 259 patients). Dependent variable: *A. fumigatus* colonization (defined as >50% of cultures positive in 2007).

	colonized (n=61)	**not colonized (n=198)**	**simple logistic regression** *p*	OR (95% CI)	**multiple logistic regression** *p*	OR (95% CI)
age category, n (%)						
0-12 years						
(reference category) (n=106)	10 (16.4)	96 (48.5)	<0.001		0.06	
13-24 years (n=99)	33 (54.1)	66 (33.3)	<0.001	4.80 (2.2-10.4)	0.02	2.90 (1.2-7.0)
≥25 years (n=54)	18 (29.5)	36 (18.2)	<0.001	4.80 (2.0-11.4)	0.09	2.39 (0.9-6.5)
male, n (%)	27 (44.3)	112 (56.6)	0.09	0.61 (0.3-1.1)		
dF508 homozygosity, n (%)	38 (63.3)	116 (61.7)	0.82	1.07 (0.6-2.0)		
pancreatic insufficiency, n (%)	52 (85.2)	170 (85.9)	0.91	0.95 (0.4-2.2)		
CF related diabetes, n (%)	8 (13.1)	20 (10.1)	0.51	1.34 (0.6-3.2)		
FEV_1 pp, mean (SD)	63.6 (25.0)	81.5 (25.5)	<0.001	0.97* (0.96-0.99)	0.31	0.99* (0.98-1.01)
BMI Z-score, mean (SD)	-0.62 (1.00)	-0.38 (1.02)	0.11	0.80* (0.6-1.1)		
inhaled antibiotics, n (%)	40 (65.6)	49 (24.7)	<0.001	5.79 (3.1-10.8)	0.001	3.72 (1.7-8.2)
rhDNase, n (%)	34 (55.7)	66 (33.3)	0.002	2.52 (1.4-4.5)	0.58	1.21 (0.6-2.4)
chronic *P. aeruginosa* infection, n (%)	43 (70.5)	80 (40.4)	<0.001	3.52 (1.9-6.5)	0.99	0.99 (0.4-2.3)
number of hospitalizations, mean (SD)	0.67 (SD 1.3)	0.29 (SD 0.7)	0.01	1.47* (1.1-2.0)	0.27	1.20* (0.9-1.7)

*change per unit of variable

Table 2 Group differences in possible confounding factors during first year of observation period. Patients were grouped according to duration of *A. fumigatus* colonization (>50% of cultures positive for *A. fumigatus* in a given year) during the observation period (from 2002 to 2007). Group 1: 0-1 years of *A. fumigatus* colonization, group 2: 2-3 years and group 4: ≥4 years. Significant differences between 3 groups were tested with Chi^2 test for dichotomous variables and with Kruskal Wallis for non-normally distributed continuous variables or ANOVA for continuous variables.

	group 1 (n=115)	**group 2 (n=29)**	**group 3 (n=19)**	*p*
age, median (IQR)	11 (7 -18)	14 (10 - 19)	21 (13 - 29)	<0.001
male, n (%)	63 (54.8)	15 (51.7)	9 (47.4)	0.82
dF508 homozygosity, n (%)	77 (68.8)	16 (57.1)	14 (73.7)	0.41
pancreatic insufficiency, n (%)	98 (85.2)	21 (75.0)	17 (89.5)	0.33
CF related diabetes, n (%)	15 (13.0)	3 (10.3)	5 (26.3)	0.25
inhaled antibiotics, n (%)	10 (8.7)	7 (24.1)	11 (57.9)	<0.001
rhDNase, n (%)	21 (18.3)	5 (17.2)	5 (26.3)	0.68
>50% cultures + for *P. aeruginosa*, n (%)	37 (39.4)	13 (56.5)	11 (61.1)	0.12
FEV_1 pp, mean (SD)	83.9 (22.8)	73.3 (20.4)	61.3 (22.7)	<0.001
number of hospitalizations, median (IQR)	0 (0-0)	0 (0-1)	0 (0-0)	0.02
BMI z-score, mean (SD)	-0.46 (1.01)	-0.57 (1.08)	-1.10 (1.07)	0.04

With regard to the decline in FEV_1 in time, patterns were rather similar for the three groups (Figure 1). The differences in FEV_1 between the groups were explained by the investigated possible confounders (the use of inhaled antibiotics, BMI z-score, age at baseline, number of hospitalizations), rather than by the presence of *A. fumigatus* colonization. As shown in Table 3 the three groups had a significantly different estimate for FEV_1% of predicted in the unadjusted model and this difference disappeared after adjusting for confounders. Adjusting for the number of cultures performed per year did not change the difference in estimates between the three groups and this variable was therefore not included in the model. Including an interaction term between FEV_1 patterns and time, in both the unadjusted and adjusted model, failed to improve model fit significantly. This further confirms that there was no significant difference in slope of lung function between the three groups. The overall decline in FEV_1% of predicted was 1.30 percentage points per year (Table 3).

Table 3 Lung function estimates for the three groups based on the mixed model analysis, showing no statistically significant difference in lung function between the three groups after adjusting for covariates.

variable	unadjusted estimate	95% CI	adjusted* estimate	95% CI
intercept	84.73	80.73 – 88.73	104.85	99.55 – 110.15
time	-1.30	-1.75 – -0.86	-1.32	-1.75 – -0.88
A. fumigatus colonization				
group 1 (0-1 year)	0		0	
group 2 (2-3 years)	-9.93	-18.70 – 1.16	-3.08	-10.20 – 4.04
group 3 (4 years or more)	-23.41	-33.85 – -12.96	-2.25	-11.64 – 7.13

*Adjusted for baseline BMI z-score, age at baseline, number of hospitalizations, years of inhaled antibiotics use.

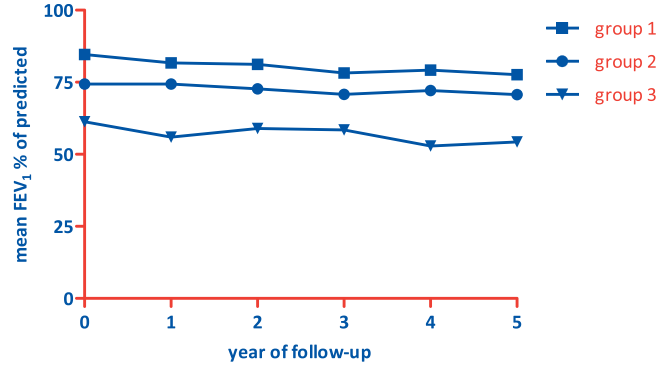

Figure 1 Mean FEV_1 % of predicted per group over the study period. Group 1 (n=115) 0-1 years of *A. fumigatus* colonization, group 2 (n=29) 2-3 years and group 3 (n=19) ≥4 years.

DISCUSSION

The results presented here suggest that colonization with *A. fumigatus*, in the absence of ABPA does not have a negative effect on the course of lung function on a population level over a five-year period.

Several other studies have investigated the association between *A. fumigatus* colonization and lung function cross-sectionally. The results from the cross-sectional analysis performed in the present study are similar to those reported by Milla et al. An unadjusted association between lung function and *A. fumigatus* colonization was found which was no longer significant after adjustment for covariates and appeared to be explained by age and gender[6], indicating that the increased risk of advanced pulmonary disease was associated with demographic factors that have been reported to be associated with severity of lung disease[18]. Another cross-sectional study by Bargon et al. also reported no significant association between *A. fumigatus* colonization (defined as at least 2 cultures positive out of a minimum of 4) and lung function, in a population of adult CF patients. Similar to our findings, a significant association with inhaled antibiotics was found[11]. The authors suggest that the use of prophylactic antibiotics may have predisposed the patients to *A. fumigatus* colonization. This has also been suggested from a study on the use of inhaled tobramycin, in which the isolation of *A. fumigatus* was increased in the treatment group, at the end of the study period[19]. Based on our findings, it is difficult to say whether there is a causal relationship between the use of inhaled antibiotics and *A. fumigatus* colonization,

since the association was already present at the start of the observation period. However, since logistic regression analysis of the cross sectional results showed an independent association between the presence of *A. fumigatus* (even after correction for severity of lung disease by correcting for FEV_1) and the use of inhaled antibiotics, a causal relationship seems possible.

Since causality between colonization and deterioration of lung function cannot be inferred from a cross-sectional analysis, we also performed a longitudinal study. Patients who are chronically colonized throughout the study period had lower lung function when compared to patients who were colonized for less than 4 years during the study period. However, this difference is no longer statistically significant after adjusting for confounders. Most importantly, there was no statistically significant difference in decline of lung function between the three groups of patients, suggesting that, within a time frame of five years, *A. fumigatus* colonization does not determine the decline of lung function. These findings differ from a recent longitudinal analysis in children with CF. Amin et al. also found that patients with persistent *A. fumigatus* infection had lower lung function than patients who were not persistently infected (defined as 2 or more positive cultures per year)[14]. However, it was not reported whether there was a difference in decline between these patients.

The possibility of detection bias is a limitation of our study. More severely affected patients are more likely to visit the CF center, more likely to produce sputum and, therefore, more likely to be sampled. This was partly accounted for by defining *A. fumigatus* colonization as a proportion of all cultures performed in a year (>50% positive). Furthermore, the mean number of cultures performed per year was corrected for in the model, but because this did not change the difference in lung function estimate between the three colonization groups, this factor was removed. Another limitation is the possibility of incomplete collection of respiratory culture data, since some patients are not exclusively under treatment at the CF center and are sometimes seen in the outpatient departments of non-CF center hospitals. Furthermore, in the present study we excluded patients with ABPA, because the aim of the study was to look at the effect of *A. fumigatus* colonization in the absence of ABPA. However, we did not include measurements of specific antibodies to *A. fumigatus* in our analysis, which means that there could have been patients who did not fulfil the criteria of ABPA, but who were sensitizing to *A. fumigatus*. Since sensitization to *A. fumigatus* has been shown to be associated with deterioration of lung function[12], this could have influenced the data.

The difficulty in studying the effect of *A. fumigatus* colonization in the present study lies in the fact that from the start of the observation period the three groups differed on many aspects: patients had worse lung function, increased age and decreased nutritional status with increased duration of colonization, similar to findings from the cross-sectional analysis. It is not clear whether these patients had decreased lung function because they had been colonized before start of the observation period, or whether they were colonized because of more severely damaged lungs. The latter explanation would be in accordance with the finding that colonization with *A. fumigatus* is uncommon in young children and initial colonization occurs at a later age than colonization with bacterial pathogens like *S. aureus* and *P. aeruginosa*[20]. In the present study, more insight into the causality of *A. fumigatus* colonization has been obtained by analyzing longitudinal follow-up data and evaluating potential confounders. The results suggest that since there is no effect of prolonged colonization on course of lung function during the observation period, the decreased pulmonary function at baseline is not likely to have been caused by *A. fumigatus* colonization. However, similar to some other studies, *A. fumigatus* was more frequently found in patients with more severe lung disease and with increased treatment burden. Therefore, although an effect on decline of lung function in this study could not be found, further prospective study is needed to definitively study causality between *A. fumigatus* colonization and treatment burden and pulmonary exacerbations and the effect of antifungal treatment on clinical outcome in colonized patients.

CONCLUSION

This study shows that patients with *A. fumigatus* colonization have less favorable clinical status and have a greater burden of treatment. However over a 5-year period, colonization with *A. fumigatus* in the absence of ABPA is not independently associated with a more severe decline in lung function.

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Chapter 5

TRANSMISSION OF PSEUDOMONAS AERUGINOSA GENOTYPES DURING A ONE-DAY OPEN-AIR EVENT FOR CYSTIC FIBROSIS PATIENTS

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SUBMITTED

Abstract

Background

The occurrence of several transmissible Pseudomonas aeruginosa clones in cystic fibrosis (CF) centers around the world has led to implementation of infection control policies to limit the spread of such clones. Patient-to-patient transmission has been reported to occur in those visiting summer camps for CF patients and the discontinuation of summer camps was implemented in 2005 in the Netherlands. Since that time, a group of young adult CF patients have organized a yearly one-day open-air patient event at the beach. We aimed to study the risk of cross-transmission during this event.

Methods

Sputum or cough swab samples were collected from patients visiting the CF Beach Dance (CFBD) event. Sampling took place upon entering the event and when patients left. Three months after the event, a final sample was collected. Data was collected regarding adherence to hygiene rules. Isolated P. aeruginosa were typed using Multiple Loci Variable number tandem repeat Analysis (MLVA). Transmission was defined as the presence of an MLVA type (MT) in the second sample, that was not present in the first sample and that could be found in another patient.

Results

Twenty-two out of 25 CF patients visiting the event participated in this study. All patients had samples obtained upon arrival at the event and again upon leaving. Follow-up samples were complete for 21 patients (95%). Culture positivity for P. aeruginosa varied from 64% to 76%. All patients were familiar with hygiene rules and 17 (77%) patients indicated they adhered to these rules to some extent while five (23%) patients indicated they completely ignored the rules. In total, 18 different MTs were found of which MT27 was the most prevalent: 36% of participants harbored MT27, that was previously linked to the highly prevalent Dutch CF clone ST406. There was one case of possible cross-transmission in which the acquired genotype (MT27) was found immediately

after the event, that was also present in other participants. Of patients with complete and positive cultures, 79% were colonized by the same P. aeruginosa MT *throughout this study.*

Conclusion

One case of possible cross-transmission was identified. However, the small amount of patients and the presence of the highly prevalent clone preclude drawing conclusions on the true risk of P. aeruginosa transmission in this setting.

Introduction

In the first two decades of life 60-80% of patients with cystic fibrosis (CF) become chronically infected with *Pseudomonas aeruginosa*. It was previously thought that patients with CF acquired individual *P. aeruginosa* strains from the environment[1;2] and sharing of *P. aeruginosa* genotypes between patients occurred only in patients within the same household[3]. In the 1990's however, several studies suggested that cross-transmission could also occur between non-related patients who visited camps for CF patients together. A retrospective study in Denmark showed that five *P. aeruginosa* negative children, who participated in a CF summer camp together with 17 *P. aeruginosa* positive children, became colonized with *P. aeruginosa*. The authors advised segregation of patients according to *P. aeruginosa* infection status[4]. In the Netherlands Brimicombe et al. performed a study in 80 children visiting a CF summer camp in 2001. AFLP typing showed 18 cases of possible transmission of *P. aeruginosa* strains between patients[5]. Several studies from CF centers in the UK and Australia reported the occurrence of highly transmissible strains of *P. aeruginosa*[6-9] and a possible detrimental effect on CF lung disease. In the Dutch CF centers' infection control policies aimed at limiting the spread of such strains were implemented in 2005 which included the discontinuation of CF summer camps and advising CF patients against meeting each other[10].

In 2005 a group of teenage CF patients started organizing the CF Beach Dance event (CFBD), an opportunity for patients to still meet each other. This one-day open-air event at the beach in Bloemendaal, the Netherlands has been organized once yearly ever since and consists of an afternoon part with physical activities and an evening part with music and dancing. The organizers chose an event of short duration in the open air based on the idea that this would minimize the risk of cross-transmission. Also, visitors are encouraged to adhere to hygiene rules, established by the Dutch CF foundation[11] (and based on guidelines from the American CF foundation). These include directions for cough hygiene and distance to other patients. The risk of cross-transmission in such a setting however has not yet been investigated, as previous studies only investigated the risk at camps of several days' duration. We hypothesized that the risk of cross-transmission of *P. aeruginosa* between patients in the CFBD setting is minimal. We aimed to study this by genotyping *P. aeruginosa* isolates obtained from

patients who visited CFBD. Furthermore, we aimed to study if *P. aeruginosa* genotype influences the risk of transmission and whether other risk factors can be identified.

METHODS

PARTICIPANTS

This study was performed during CFBD 2009, which took place on July 18th 2009, in Bloemendaal, the Netherlands. CFBD is promoted through a website, which also gives information about the background of the event and about hygiene precautions. Potential visitors were informed of the study via the event's website, on which CF patients are encouraged to register for the event (the event is open to non-CF patients, but they are not asked to register). Those who registered online were asked to participate in the study, and informed consent from the patients (and their parents if applicable) was obtained. Patients who had not registered online, but who had been informed about the study online, were recruited upon arrival to the event and informed consent was obtained on site. The protocol for this study was approved by the local institutional review board (UMC Utrecht, protocol number 08/421).

SAMPLING

Upon arrival at the beach, before being transported to the festival terrain, patients were asked to complete a short questionnaire with their personal details and some information on their current treatment, and a sputum sample was collected by the patients themselves (t1). If patients were unable to produce sputum, a cough swab was collected by a researcher. Sampling was repeated when patients left the festival terrain (t2), and the patients completed a short questionnaire on their familiarity with and adherence to the hygiene rules. Three months after CFBD, a sampling package was sent to the participants via mail (t3). The package included material for sputum and cough swab collection with an instruction manual for how to obtain the samples and a short survey on whether patients had met up with other CF patients since CFBD.

ISOLATION OF BACTERIA AND TYPING

Cough swabs that were obtained at the event were immediately streaked on agar plates and sputum samples were processed the next day. All respiratory samples were cultured according to standard laboratory practice and

P. aeruginosa isolates were frozen for storage. Multilocus variable number tandem-repeat analysis (MLVA) was performed according to the 'MLVA9-Utrecht' typing scheme as described before[12].

TRANSMISSION

Transmission was defined as the finding of a *P. aeruginosa* strain, that does not belong to the MLVA types (MTS) present in the sputum of the patient upon arrival to the event (t1) but was present at t2, and was also found in another participant at t1[5].

RESULTS

PARTICIPANTS AND SAMPLES

Out of 25 CF patients visiting the event, 22 CF patients agreed to participate in this study. Table 1 displays baseline characteristics of the participants. All patients had samples obtained upon arrival at the event and again upon leaving. Follow-up samples were complete except for one patient who did not send a third sample. Culture positivity for *P. aeruginosa* varied from 64% to 76% (Table 2). When asked about the hygiene rules, all patients were familiar with these rules and 17 (77%) patients indicated they adhered to these rules to some extent (1 'strictly' and 16 patients 'more or less') while 5 (23%) patients indicated they completely ignored the rules.

Table 1 Participant characteristics at day of event

age, mean (SD)	28	(11)
male, n (%)	10	(45)
using antibiotics, n (%)	18	(82)
inhalation	9	(41)
oral	18	(82)
history of lung transplant, n (%)	6	(27)
time since last hospitalization, n (%)		
less than 3 months	4	(18)
between 3 months and 1 year	7	(32)
more than 1 year	10	(46)

Table 2 Sample types and *P. aeruginosa* positivity

	t1	t2	t3
cough swab	11 (50%)	13 (59%)	7 (33%)
positive	3 (27%)	7 (54%)	4 (57%)
sputum	11 (50%)	9 (41%)	14 (67%)
positive	11 (100%)	9 (100%)	12 (86%)
total	22	22	21
positive	14 (64%)	16 (73%)	16 (76%)

Of the 22 participants, there were 3 pairs of patients who shared a household and there were 3 patients who shared a household with (an)other CF patient(s) not present at the event. Three months after the event, when asked about contact with other CF patients, 11 (50%) patients indicated they had had contact with another CF patient other than their sibling or household contact.

MLVA TYPES

In total, 131 morphologically different *P. aeruginosa* strains were isolated from 16 patients at three time points. After exclusion of identical MTS in each culture, 59 strains remained belonging to 18 different MTS (Table 3). Three MTS were shared between patients (MT 11, 27 and 98). MT27 was the most prevalent type in 36% (8) of the patients. This MT is linked to the highly prevalent *P. aeruginosa* clone ST406 reported previously among CF patients in the Netherlands[13]. In total there were nine patients with a newly acquired MT at t2 or t3 that was not present in that patient at t1. Of these patients, there were two patients (patient 2 and 14) whose newly acquired MT was also found in other patients at t1. Patient 2 did not fulfill our criteria for transmission, since the MT was first cultured at t3, three months after the event. Patient 14 however, acquired a new MT at t2 (MT27), that was present at t1 in other patients, thus fulfilling the criteria for cross-transmission at the event. The newly acquired MT was also isolated at t3 suggesting that *P. aeruginosa* acquisition resulted in sustained and not only transient colonization. Both these patients had indicated to have more or less followed the hygiene rules (Table 3). Most patients (79% of patients with complete and positive cultures) were colonized by the same *P. aeruginosa* MT throughout this study.

Table 3 MLVA types (MT), adherence to hygiene rules and contact with other CF patients. Red squares represent negative cultures. Multiple MTS in one square represent single locus variants (SLV). NC=not cultured.

Patient*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
MT t1							44										293					
		27	98	27	27		296		300		27		27	230	27		403			399	398	27
MT t2			98	298			295		300					27			293					
			395	27	27		392		400		27		27	230	27		403			399	398	27
MT t3					11		296		300								293					
	NC	98	98	27	27		392		400		27		27	27	27				13	399	398	27
hygiene rules**	2	2	2	2	2	1	2	2	2	2	1	1	2	2	2	1	2	3	2	2	1	2
household contacts	-	+	-	-	-	-	-	+	-	+	-	+	+	+	+	-	-	-	-	-	-	+
other CF patients	-	+	+	-	+	-	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	+

*Underscored patient numbers represent patients with a history of lung transplant.
**Their adherence to hygiene rules was indicated by the patients as follows:
1 Hygiene rules were ignored completely;
2 Hygiene rules were more or less followed;
3 Hygiene rules were very strictly adhered to.

DISCUSSION

In this study on the transmission risk during an open-air event for CF patients, one case of possible transmission was identified. There were seven other patients with newly obtained strains, but these MTS could either not be identified in other participants (six patients) or the MT was only first cultured three months after the event (one patient). Although some of these cases might represent transmission of a strain acquired from a CF patient who did not participate in this study, this is not likely since there were only three non-participating CF patients who visited the event. In six patients, including one possible transmission case (patient 14), the new MTS found were Single Locus Variants (SLVs) of types already found in that patient. Possibly this represents in-patient evolution of the chronically colonizing *P. aeruginosa* strains, where SLVs of the initial colonizing strain are simultaneously present. This is supported by the fact that in 9 cultures (20% of all positive cultures) SLVs were present simultaneously. Our results show that many of the participants still have contact with other CF patients (beside their household contacts), as 11 (50%) patients indicated they had had contact with another CF patients in the three months after CFBD. Also, although the majority of patients adhered to the hygiene rules, which the CFBD organization encourages patients to follow, to some extent, only one patient followed these rules 'strictly.'

Among the participants approximately one third of patients were free of *P. aeruginosa*. This could partly be explained by the fact that three out of the seven negative patients had had a lung transplant, which explains why they were no longer infected with *P. aeruginosa*. It was unexpected however that uninfected patients visited this event, since for these patients the risk of acquiring *P. aeruginosa* could possibly make these patients less willing to meet with other CF patients.

A high number of patients was colonized with MT27. This clone has been previously identified as a highly prevalent clone in Dutch CF patients and is designated ST406 upon MLST typing. This *P aeruginosa* clone is thus far only found in CF patients and not in patients in the ICU or on other wards[12]. Previous transmission of this clone at social events (like summer camps) or in the hospital seems likely although in this limited study we found no cases of possible transmission of this clone during the CFBD. It is possible that several potential cases of transmission are missed; since our analysis is based on genotyping we cannot distinguish between MT27 from different patients. It is possible that MT27 clones were cross-transmitted between patients who were already

harboring strains belonging to MT27. The use of phenotypic assays in addition to genotyping could be suggested in order to identify transmission. However, the large within-patient variability of phenotypic traits, even for strains of the same genotype[14], makes this a less suitable method, which requires testing of many isolates per sample. Still, it could be important to further study whether the transmissibility of certain clones is determined by specific phenotypic traits. It could be hypothesized that only certain subpopulations of *P. aeruginosa* strains are capable of cross-transmission. So far, a specific phenotypic trait responsible for the increased transmission of certain clones has not been identified.

It can also be argued that the possibility of transmission from the environment or healthy visitors to CFBD to CF patients should be taken into account, in order to investigate the risk of cross-transmission of *P. aeruginosa*. Healthy people are known to sometimes carry *P. aeruginosa* strains temporarily, for instance during respiratory virus infections[15]. Our aim however was, to investigate whether there is an increased risk of cross-transmission between CF patients, in order to advice them on whether or not to go to such an event where other CF patients will be. We did not aim to study the general risk of acquiring *P. aeruginosa* from healthy people or the environment.

As previous editions of CFBD had seen increasing numbers of visitors, the estimated minimal number of participants was fifty. However, due to heavy winds and rain on the day of the event there were fewer visitors than expected. Therefore, the results from this study should be interpreted with care; the low number of participants precludes drawing definite conclusions on the risk of *P. aeruginosa* transmission during the one-day open-air CF Beach Dance event and the influence of genotypes on transmission rate. Also, due to small numbers it was not possible to statistically analyze whether there were certain risk factors associated with transmission. Because of the weather, a large part of the day was spent indoors at the beach club, while one of the main reasons for a beach event is that participants can be outdoors, to possibly minimize a risk of cross-transmission. The few participants, and the partly indoor character, make this not a representative setting of this event.

In conclusion there is one case of possible cross-transmission. However, the small amount of patients and the presence of the highly prevalent clone preclude drawing conclusions on the true risk of *P. aeruginosa* transmission in this setting.

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Chapter 6

CLINICAL IMPACT OF A HIGHLY PREVALENT PSEUDOMONAS AERUGINOSA CLONE IN DUTCH CYSTIC FIBROSIS PATIENTS

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ADAPTED FROM CLINICAL MICROBIOLOGY AND INFECTION 2011 MAR;17(3):382-5.

Abstract

Background

The occurrence of highly prevalent Pseudomonas aeruginosa clones reported in cystic fibrosis centers around the world has led to implementation of strict infection control measures. Studies suggest that high prevalence of P. aeruginosa strains is associated with unfavorable clinical outcome. Recently a highly prevalent P. aeruginosa clone was found in patients of two CF centers in the Netherlands, designated ST406. We aimed to study whether infection with this clone was associated with unfavorable clinical outcome.

Methods

Respiratory tract infection status was determined in 512 CF patients in 2007. Demographic, genetic and clinical parameters were obtained cross-sectionally. P. aeruginosa positive patients were designated as infected with either ST406 or other sequence types. Logistic regression was performed to study factors associated with ST406 infection.

Results

In total, 265 patients (52%) were chronically infected with P. aeruginosa. MLST data were available for 219 patients, of whom 40 (18.4%) were infected with ST406 and 179 were infected with other sequence types. Although chronic P. aeruginosa infection was independently associated with decreased lung function, infection with ST406 was not associated with decreased lung function, increased number of hospitalization days or decreased nutritional status. ST406 infection was independently associated with age (OR 24.83, p=0.005 for age 13-24 years compared to age 1-12 years), having a sibling with ST406 (OR 20.17, p=0.001) and use of inhaled antibiotics (OR 3.42, p=0.03).

Conclusion

Unlike other reported highly prevalent P. aeruginosa clones, infection with ST406 is not associated with unfavorable clinical outcome in this population, suggesting that high transmissibility is not necessarily associated with high virulence.

Introduction

In cystic fibrosis (CF) 60-80% of patients become chronically infected with *Pseudomonas aeruginosa*[1], which is associated with increased morbidity and mortality[2;3], The original perception that each patient acquires his own *P. aeruginosa* strain from the environment and that transmission only occurs in siblings with CF[4] is disputed by later reports of the occurrence of highly prevalent clones of *P. aeruginosa* in CF centers in Australia and Europe[5-11].

Most of the reported widely distributed clones are highly resistant to antibiotics and appear to be associated with increased treatment requirements[12] and hospitalization[8] or increased morbidity[13]. In order to prevent the further spread of transmissible *P. aeruginosa* clones, infection control policies and measures have been put into practice in several countries[14;15]. In the Netherlands, all CF patients are seen and treated separately in both the inpatient and outpatient setting. Furthermore, patients are advised to limit contact with other CF patients outside the hospital and the organization of summer camps for CF patients has been discontinued[16].

Recently, a cross-sectional study on the population structure of *P. aeruginosa* within CF patients of the CF centers of Utrecht and The Hague in The Netherlands was performed[17]. Together these centers manage around 45% of the total Dutch CF population. Multi Locus Sequence Typing (MLST) of *P. aeruginosa* isolates revealed the occurrence of a highly prevalent clone in this patient population, designated ST406. Genetically this clone was not found to be closely related to any of the earlier described 'epidemic' *P. aeruginosa* clones and it appears not to be characterized by a specific antibiotic resistance profile. It is unclear whether this highly prevalent clone is associated with unfavorable clinical outcome. We therefore aimed to study the clinical status of patients chronically infected with *P. aeruginosa* and to study whether infection with ST406 is associated with unfavorable clinical outcome in the CF patient population of the CF centers of Utrecht and The Hague, The Netherlands.

METHODS

PATIENTS AND SAMPLE COLLECTION

This study was performed in patients under treatment of the CF centers of Utrecht and the Hague in 2007. In both centers, segregation policies have been adopted since 2005. Sputum samples and throat swabs were collected regularly and were cultured using standard diagnostic laboratory protocols of each CF center. MLST was performed on all phenotypically different isolates of the first *P. aeruginosa* positive culture from each patient in 2007 (Utrecht) and the first half of 2008 (the Hague) as described in a previous study[17].

Patients were designated as chronically infected if *P. aeruginosa* was present in more than 50% of all cultures (sputum or oropharyngeal) collected in 2006 and 2007[18]. Patients were designated as infected with the highly prevalent clone (ST 406) or as infected with other sequence types. Patients carrying a combination of multiple sequence types including ST406 were regarded as ST406 carriers.

DATA COLLECTION

Demographic data and CF genotype were collected from the centers' databases. Data regarding presence of complicating subdiagnoses (pancreatic insufficiency, nasal polyposis, allergic bronchopulmonary aspergillosis (ABPA), CF related liver disease, distal intestinal obstruction syndrome (DIOS) and CF related diabetes were collected. Additionally, the use of inhaled antibiotics (colistin, tobramycin or both) and the use of recombinant human DNase (rhDNase) were recorded for 2007. The presence of other microorganisms in the respiratory cultures used for typing as well as the number of hospitalization days for the previous year was recorded. Lung function (Forced Expiratory Volume in 1 second (FEV_1) % of predicted) and height and weight measurements were recorded during the annual multidisciplinary check-up visit to the CF clinic or during a routine clinic visit. BMI (Body Mass Index) Z-scores were calculated based on growth curves for either Dutch, Turkish or Moroccan children where appropriate[19]. All patients gave written informed consent to store and evaluate their clinical data in the CF-database for scientific purposes.

STATISTICAL ANALYSIS

Simple logistic regression was performed to evaluate the association between the study variables and the dependent variable (chronic *P. aeruginosa* infection and ST406 infection). In order to study which variables were in-

dependently associated with the outcome, variables showing an unadjusted association (p<0.20) were entered into a multiple logistic regression model. The number of factors included in the model was limited to ensure at least 10 outcome events per independent variable[20]. This was done by taking into account the variables with the strongest association in the simple logistic regression. Variables were entered into the multiple model using the 'enter' method. Differences between categorical data were analyzed using Fisher's Exact Test. Statistical analyses were performed using SPSS for Windows version 15.0.1 (SPSS, Chicago, Illinois, USA).

RESULTS

In total 561 patients were under treatment at the CF centers of Utrecht (355) and The Hague (206) in 2007. Cultures (sputum and throat swab samples) were collected from 515 patients (92%) during that year. Ten patients were excluded from the analysis because they were chronically infected with *B. cepacia complex*. Of the 505 patients included in the analysis, 265 patients (52%) were chronically infected with *P. aeruginosa*. MLST data were available for 219 patients with chronic *P. aeruginosa* infection (12 patients had negative *P. aeruginosa* cultures in 2007, 20 patients had isolates that were not available for typing and 14 patients had an isolate that was not typable). Of these patients, there were 40 (18.3%) patients with ST406 and 179 patients with other sequence types (not ST406).

CF genotype data were available for 472 (94%) out of 505 patients included in the analysis. Lung function data were available for 469 (93%) patients: lung function was not measured at the CF center in 7 cases and in 29 cases the patient was too young for lung function measurement. Age at diagnosis was available for 478 (95%) patients and there were 2 patients in whom BMI was not recorded.

Results of the logistic regression analysis of factors associated with chronic *P. aeruginosa* infection are displayed in Table 1. In the multiple logistic regression model, 445 out of 512 patients were included (67 patients were not included because of missing lung function data (34), missing CF genotype (31) or both (2)). Factors independently associated with chronic *P.aeruginosa* infection are increased age (OR 3.69 and 2.31 for 13-24 and ≥25 years age categories respectively, p<0.001 and p=0.02), use of inhaled antibiotics (OR 7.38; p <0.001) and lower FEV_1% of predicted (OR 0.98 per unit of variable; p =0.003).

Factors associated with infection with the highly prevalent clone ST406 are shown in Table 2. An unadjusted association was found with younger age, with being under treatment at the CF center in Utrecht and with having a sibling with ST406. There was no significant association between sequence type and FEV_1% of predicted, BMI and the number of hospitalization days. To ensure 10 outcome events per independent variable, 4 variables were selected (infection with ST406 was present in 40 cases). The 3 variables with the strongest association in the simple logistic regression analysis were included (sibling with ST406, age and CF center) together with the use of inhaled antibiotics, since this variable had a borderline p value (0.05) it was included in the model. The use of inhaled antibiotics (OR 3.42; p=0.03), age (OR 24.83 for age 13-24 years compared to 1-12 years, p=0.005) and the presence of a sibling with ST406 (OR 20.27; p =0.001) were independently associated with infection with ST406.

In order to evaluate whether the strong association with having a sibling with the same *P. aeruginosa* sequence type was a unique feature of ST406, siblings (who were living together at the time of study) were evaluated for sequence type concordance. Among the patients with a positive *P. aeruginosa* culture, were 29 sibling pairs or trios. There were 7 pairs and 1 trio with at least 1 person carrying ST406, and of which all members were cultured in 2007. Of these siblings, 6 pairs/trios (75%) were concordant for ST406 (at least two persons were infected with ST406). There were 17 sibling pairs and 2 trios with at least 1 sibling infected with a sporadic ST and of which all members were cultured. Of these siblings, there were 11 pairs/trios (58%) with at least 2 siblings carrying the same ST. The difference between the proportions of concordant siblings was not significant between ST406 and sporadic sequence types (p=0.67).

Table 1 Association between clinical and demographic variables and chronic *P. aeruginosa* infection (results of logistic regression analysis) in 505 CF patients.

	simple logistic regression				multiple logistic regression	
	OR (95% CI)	Pseudomonas-positive (n=265)	Pseudomonas-negative (n=240)	*p*	OR (95% CI)	*p*
male gender	0.85 (0.60-1.21)	133 (50.2%)	130 (54.2%)	0.37		
age (years)						
1-12 years (reference category)		42 (15.8%)	118 (49.2%)	<0.001		<0.001
13-24 years	5.20 (3.21 – 8.44)	100 (37.7%)	54 (22.5%)	<0.001	3.69 (1.98 – 6.88)	<0.001
≥25 years	5.08 (3.21 – 8.05)	123 (46.4%)	68 (28.3%)	<0.001	2.31 (1.17 – 4.57)	0.02
homozygosity for dF508	1.39 (0.96-2.01)	154 (63.4%)	127 (55.5%)	0.08	1.62 (0.99-2.64)	0.05
pancreatic insufficiency	1.02 (0.65-1.60)	217 (81.9%)	196 (81.7%)	0.95		
ABPA	2.32 (1.30-4.16)	42 (15.8%)	18 (7.5%)	<0.01	1.49 (0.74-3.00)	0.27
diabetes	2.26 (1.38-3.70)	59 (22.3%)	27 (11.3%)	<0.01	0.90 (0.47-1.70)	0.74
rhDNase	2.67 (1.84-3.88)	132 (49.8%)	65 (27.1%)	<0.001	1.15 (0.69-1.91)	0.59
inhaled antibiotics	10.66 (6.90-16.47)	173 (65.3%)	36 (15.0%)	<0.001	7.38 (4.46-12.19)	<0.001
hospitalization days	1.02* (1.01-1.04)	7.59 (SD 16.7)	3.50 (SD 13.2)	<0.01	1.00* (0.99-1.02)	0.82
BMI Z-score	0.92* (0.78-1.09)	-0.40 (SD 1.1)	-0.31 (SD 1.0)	0.35		
FEV^1 (% of predicted value)	0.97* (0.96-0.98)	63.78 (SD 24.0)	82.39 (SD 24.2)	<0.001	0.98* (0.97-0.99)	0.003

*Change per unit of variable.

Table 2 Association between clinical and demographic variables and ST406 infection (results of logistic regression analysis) in 219 chronically infected cystic fibrosis (CF) patients

	Simple logistic regression				Multiple logistic regression	
	OR (95 % CI)	ST406 (n=40)	Other ST (n=179)	*p*	OR (95% CI)	*p*
CF center Utrecht (vs. CF center The Hague)	3.16 (1.38-7.24)	32 (80.0%)	100 (55.9%)	<0.01	2.13 (0.70-6.41)	0.18
male gender	0.97 (0.49-1.92)	20 (50.0%)	91 (50.8%)	0.92		
age (years)						
1-12 years (reference category)		1 (2.5%)	32 (17.9%)	<0.001		<0.001
13-24 years	23.68 (3.09 -181.25)	37 (92.5%)	50 (27.9%)	0.002	24.83 (2.59 – 237.73)	0.005
≥25 years	0.66 (0.06 – 7.52)	2 (5.0%)	97 (54.2%)	0.74	1.16 (0.08 – 16.61)	0.91
homozygosity for dF508	1.93 (0.88-4.22)	30 (75.0%)	98 (60.9%)	0.10		
sibling with ST406	42.61 (9.11-199.32)	13 (32.5%)	2 (1.1%)	<0.001	20.27 (3.62-113.58)	0.001
pancreatic insufficiency	1.10 (0.42-2.85)	34 (85.0%)	150 (83.8%)	0.85		
ABPA	0.35 (0.10-1.19)	3 (7.5%)	34 (19.0%)	0.09		
diabetes	1.01 (0.44-2.29)	9 (22.5%)	40 (22.3%)	0.98		
rhDNase	0.89 (0.44-1.76)	18 (45.0%)	86 (48.0%)	0.73		
inhaled antibiotics	2.28 (0.99-5.24)	32 (80.0%)	114 (63.7%)	0.05	3.42 (1.14-10.24)	0.03
hospitalization days	1.01* (0.99-1.03)	10.8 (SD 26.9)	7.4 (SD 14.6)	0.28		
BMI Z-score	0.91* (0.67-1.23)	-0.5 (SD 1.2)	-0.4 (SD 1.1)	0.53		
FEV^1 (% of predicted value)	1.01* (1.00-1.02)	67.3 (SD 27.3)	61.9 (SD 23.5)	0.20		

*Change per unit of variable

DISCUSSION

Our cross-sectional study shows that infection with a highly prevalent *P. aeruginosa* clone, ST406, is not associated with an unfavorable clinical status in patients with CF. The presence of a sibling with ST406, age and the use of inhaled antibiotics are independently associated with infection with ST406. The transmissibility of ST406 in siblings is comparable to other sequence types.

In general, chronic infection with *P. aeruginosa* is independently associated with a severe CF genotype, decreased pulmonary function and with the use of inhaled antibiotics. These results in a population of the two largest CF-centers in The Netherlands are in accordance with the literature[2;21;22]. However, the findings regarding the Dutch clone ST406 are different from those described in other countries. The Australian Epidemic Strain 1 (AES-1) was associated with increased hospitalization and rhDNase use, and with decreased lung function and clinical score[8]. Patients harboring the Australian Epidemic Strain 2 (AES-2) had poorer lung function. However, treatment requirements were not different for patients infected with AES-2[23]. Patients carrying a highly transmissible strain in the Manchester CF center had greater treatment requirements, including more inpatient days and increased use of intravenous antibiotics[12]. A study on chronic infection with the Liverpool Epidemic Strain (LES) showed greater decline of both lung function and nutritional status over a five-year period[13]. However, upon longitudinal analysis over an 8-year period, there was no difference in survival, lung function or BMI deterioration between patients with this clone and those without it[24]. There was an increased healthcare utilization in patients with the clone, which could have been due to increased treatment requirements of the patients harboring the clone, but it could also be explained by the physician's awareness of the patient's infection status. Only one study found a difference in use of inhaled antibiotics, but this was contrary to our results, since infection with this highly prevalent clone was associated with less frequent use of inhaled antibiotics[25]. The authors suggested this could be due to increased resistance of the clone, but two other studies of multiresistant clones have not shown a difference in use of inhaled antibiotics between clonal and sporadic strains[8;23]. The explanation for an association between infection with ST406 and increased use of inhaled antibiotics can only be speculated on. A possible explanation could be increased persistence of ST406 during treatment with inhaled antibiotics. Another important difference between the clones reported in literature and ST406 investigated in the present study is the lack of a specific antibiotic resistance profile in ST406[5;6;23].

This might partly explain the increased morbidity and prolonged respiratory exacerbations in patients with reported widely distributed clones.

Since ST406 was not associated with unfavorable outcome in the present study, it appears that ST406 is, unlike the other reported highly prevalent clones[26-28], not characterized by increased virulence. This corroborates findings from in vitro studies of two highly prevalent clones in Copenhagen, where both highly prevalent clones displayed decreased virulence in a *C. elegans* virulence assay[29]. Analogous to these findings, the presented results of ST406 suggest that ST406 might be another clone that is highly transmissible but not highly virulent. Laboratory studies are needed to investigate the in vitro characteristics of ST406.

An important finding is that ST406 was most strongly associated with having a sibling with ST406, pointing to high transmissibility of this strain within families. However, this appeared not to be unique for ST406 but also true for other clones, illustrating the general importance of household transmission for the spread of *P. aeruginosa* between patients.

A limitation to our study is the cross-sectional nature, which raises questions about the duration of ST406 infection. It is possible that ST406 was only introduced into the studied CF population recently, and therefore has only shortly been able to exert a possible effect on clinical status. However, in a 2001 study on the transmission of *P. aeruginosa* during summer camps for CF patients in the Netherlands[30], the most prevalent Amplified Fragment Length Polymorphism type found, was found to be ST406 upon MLST typing[17]. These results suggest that ST406 has been present in the Dutch CF population for at least six years. Furthermore, since the segregation policy was adopted in 2005 it seems most plausible that the spread of ST406 took place before 2005.

Of all the patients with typed *P. aeruginosa* isolates, 26 had more than one sequence type in their sputum culture. Among these, 10 patients carried ST406. In the analysis, these patients were designated as ST406 carriers, since we aimed to investigate the factors associated with infection with ST406, regardless of whether or not there were other STs in the same sputum or throat swab sample. This could have influenced the data, if the sporadic STs had any association with the investigated variables. Therefore an additional analysis was performed, from which these 10 patients were omitted, yielding similar results (data not shown).

In conclusion, in the present study we have cross-sectionally studied the association between a highly prevalent *P. aeruginosa* clone, designated ST406, and clinical status and treatment burden in the population of two large CF centers in the Netherlands. Although chronic infection with *P. aeruginosa* was associated with decreased lung function, infection with ST406 was not associated with unfavorable clinical outcome in this population which suggests that ST406 is, unlike other reported widely distributed clones, not highly virulent.

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Chapter 7

INCREASED PERSISTENCE OF A DUTCH PSEUDOMONAS AERUGINOSA CLONE FOUND IN CYSTIC FIBROSIS PATIENTS

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SUBMITTED

Abstract

Background

The occurrence of highly prevalent clones of Pseudomonas aeruginosa in patients with cystic fibrosis has been reported. Besides patient-to-patient transmission, increased capacity to persist might contribute to the success of such clones. We aimed to study whether the recently discovered Dutch highly prevalent clone was associated with increased persistence. Furthermore, we aimed to study whether infection with the highly prevalent clone was associated with adverse clinical outcome.

Methods

We performed a follow-up study of a P. aeruginosa transmission study performed in 2001 during a CF summer camp. Participants still alive in 2010 were invited to send sputum or cough swab samples. Isolates from 2001 and 2010 were typed using multi locus variable-number tandem-repeat analysis (MLVA) and clinical data from 2001-2010 were collected. The risk of lung transplantation or death was in vestigated using a Cox proportional hazard model and lung function decline was investigated using a linear mixed model analysis.

Results

Of the 80 participants in 2001, 66 patients were alive in 2010 and 41 participated in the study. P. aeruginosa isolates belonging to clonal complex 27 (CC27) were found in 18 patients (44%) in 2001 and in 20 patients (49%) in 2010. Persistence of CC27 isolates was significantly higher than of other isolates (83% and 48% respectively, p=0.018). CC27 was not associated with an increased risk of lung transplantation or death (adjusted HR 1.01, 95% CI 0.41 – 2.49), and there was no significant difference in lung function decline when comparing patients persistently infected with CC27 and other patients.

Conclusions

This study shows that the Dutch highly prevalent P. aeruginosa clone displays increased persistence over a 9-year period when compared to other sporadic clones. However, no association with adverse clinical outcome was found.

Introduction

In cystic fibrosis (CF), patients become chronically infected with specific pathogens, predominantly *Pseudomonas aeruginosa*, which infects 60-80% of adult patients[1]. Although many patients are infected with unique strains that are presumably acquired from an environmental source[2-4], the occurrence of shared *P. aeruginosa* clones between unrelated patients at CF centers has been reported since the 1990's. The first reports of these highly prevalent clones showed an association with an unfavorable clinical outcome[5-8] but many of these studies were cross-sectional, and recent longitudinal studies have shown conflicting results on the effect of these clones[9;10]. Infection control policies were implemented to limit the spread of these clones. Whereas in the UK and Australia cohort segregation was implemented[8;11], in the Netherlands individual patient segregation was implemented in 2005, as well as the discontinuation of summer camps and advising against physical contact between CF patients outside the hospital[12].

A study on the transmission of *P. aeruginosa* among patients attending a CF summer camp in 2001 already showed the occurrence of a dominant *P. aeruginosa* clone[13]. This was confirmed by a large scale cross-sectional analysis of the *P. aeruginosa* population structure of patients in the two largest Dutch CF centers in 2007, using multi-locus sequence typing (MLST), which showed a highly prevalent clone designated ST406[14]. This clone was not genetically linked to any of the reported highly prevalent clones and was not characterized by a specific antibiotic resistance profile or by a risk of adverse clinical outcome[15]. The fact that 70% of patients in the cross-sectional study were infected with a genotype that was also found in other CF patients within this population, suggests an important role for patient-to-patient transmission in general[14].

Several reports suggested that highly prevalent *P. aeruginosa* clones are sometimes characterized by specific virulence factors that are thought to contribute to their success, e.g. factors that might promote transmission[16]. Besides patient-to-patient transmission, another factor that could contribute to the success of highly prevalent clones is an increased capacity to persist. Because the 2007 study showed that two years after the implementation of individual segregation, the majority of patients were colonized with a genotype that was also found in other patients[14], we aimed to investigate the persistence of the different *P. aeruginosa*. We hypothesized that ST406 shows increased persistence

over a nine-year period when compared to sporadic clones, thus contributing to its success. Furthermore, we aimed to study whether ST406 is associated with a more severe course of CF lung disease, since in contrast to other highly prevalent clones, cross-sectional data revealed no association with adverse clinical outcome[15].

METHODS

PATIENTS

This study was a follow-up of a study performed in 2001 that aimed at studying transmission of *P. aeruginosa* between patients during a summer camp for CF patients. In this study all patients visiting the summer camp were cultured on the first day of the camp, 3 months and 6 months after the camp. All participants of the 2001 summer camp study who were still alive were invited via mail and/or telephone to participate in the study. Those who agreed to study participation were sent a sample collection package by mail and were asked to send a sputum or cough swab sample to the investigators. Approximately one month after patients had returned the sample, another collection set was sent for the second sample. Patient instructions on how to collect the sputum sample or cough swab were included in the collection set. Clinical data of the participants were retrospectively collected from the medical records of all patients. The institutional ethical review board waived the need for individual informed consent.

SAMPLES

All sputum and throat swab samples were cultured by standard diagnostic laboratory protocols. The *P. aeruginosa* strains that were isolated and typed with Amplified Fragment Length Polymorphism (AFLP) in 2001 were retyped using Multiple Loci Variable number tandem repeat Analysis (MLVA) for all patients who participated in 2010 for accurate interpretation of persistence. The samples were plated on Trypticase soy agar II with 5% sheep blood (TSA-blood) plates and MacConkey agar plates (Becton, The Netherlands) to detect *P. aeruginosa*. The plates were incubated at 37°C for 2 days, after which the isolates were identified on the basis of morphology, the oxidase reaction and no inhibition on growth of C390. One *P. aeruginosa* colony of each different colony morphology (according to rough, smooth, mucoid characteristics and colony size) per sample was randomly picked and stored at -70°C for typing.

MLVA TYPING

On all *P. aeruginosa* strains from both 2001 and 2010 MLVA was performed according to the 'MLVA9-Utrecht' typing scheme as described before[17]. MLVA types (MTs) with identical number of repeats in 8 out of 9 loci (single locus variants; SLVs) were considered to belong to the same clonal complex (CC). CCs were named after their presumed founder MT, based on eBURST criteria[18]. MLVA9-Utrecht profiles were clustered with Bionumerics software (version 5.1) by using a categorical coefficient and a graphing method called minimum spanning tree.

STATISTICAL ANALYSIS

Normality of data was tested using the Kolmogorov-Smirnoff test and for normally distributed data, the Student's t-test was performed to study significant differences. For non-normally distributed data the Chi-square test or Fisher's exact test were used where appropriate. For the survival analysis, a Cox proportional hazards model was performed with lung transplantation or death as the outcome event. If patients died after having undergone a lung transplantation, the time of lung transplantation was considered as the time of censoring (and not time of death).

For longitudinal analyses of lung function, a linear mixed model was used. For patients who had had a lung transplantation, lung function measurements were only included up to the time of transplantation. In the model, persistence of the highly prevalent clone and age were included as fixed factors. The model assumed a linear trend in FEV_1 over time for each patient, and allowed for random patient-specific slope and intercept. A difference in decline of FEV_1 between the two groups was tested by examining the improvement in model fit after adding of an interaction term to the model (time*group). This interaction term would allow for different slopes over time for the two groups. All analyses were performed using SPSS for Windows, version 15.0.

RESULTS

PATIENTS

In the original study in 2001, there were 80 participants (age range 6-19 years). In 2010, 14 patients of the 80 participants had died. There were 3 patients who were still alive, but data regarding current treatment center and lung transplantation could not be retrieved. Therefore, these patients were not included in the survival analysis. Figure 1 presents a flow-chart of participant

recruitment. In total, 52 sputum samples and 19 cough swab samples were collected. Of the participants in this follow-up study, 9 received a lung transplant somewhere during the study period.

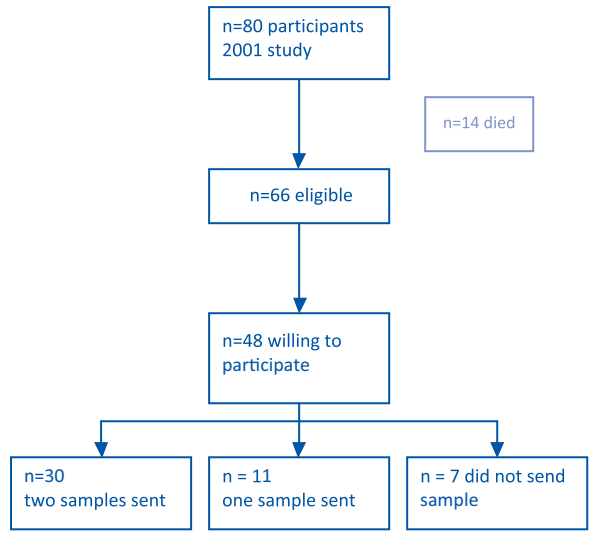

Figure 1 Flow-chart of participants

MLVA TYPES

A total of 233 morphologically different isolates were collected and typed using MLVA, (100 from 2001, and 133 from 2010). Figure 2 displays a minimum spanning tree of the MLVA types that were found in 2001 and in 2010, and graphically represents their genetic relatedness and prevalence. For each year, 1 sample per patient per type was included in the tree, yielding a total of 180 entries. There were 17 SLVs (Figure 2: short, bold connecting lines); these were assigned to the CC of the MT that was the most prevalent. Three MTs were double locus variants (DLV's) of other MTs. The 17 SLVs were found in a total of 18 patients. In these patients, when considering one isolate per MT per year, 24 SLVs were present of which in 19 cases (79%) the SLVs of the same CC could be found in the same patient. The clonal complexes or singleton MTs (not belonging to a CC) found in each patient are displayed in Figure 3, from which the dominance of CC27 becomes apparent. Isolates belonging to CC27 are those designated AFLP type 18 in the previous study[13].

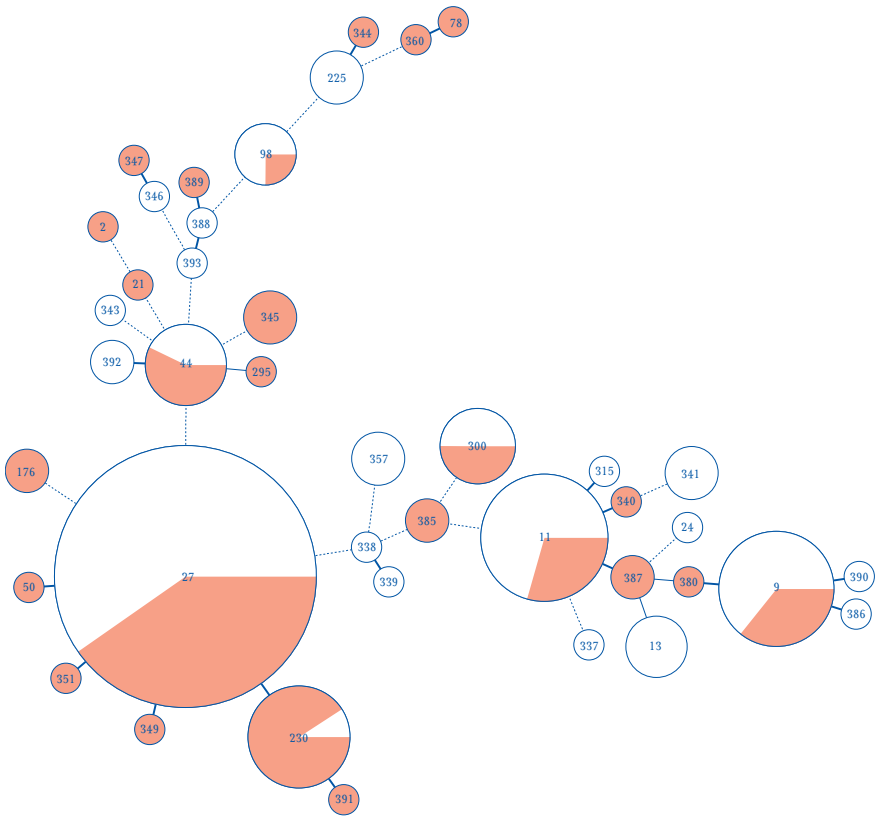

Figure 2 Minimum spanning tree representing isolated MLVA types (MT) in 2001 and 2010 and their relatedness. Each circle represents an MT and circle size represents the number of isolates. Bold lines are single locus variants (SLV) and thin lines double locus variants (DLV).

CONCORDANCE BETWEEN CULTURES

Of the 30 patients who sent two samples, concordance between the two 2010 cultures was high. In 25 patients (83%), *P. aeruginosa* positivity was similar in both cultures with 4 patients who had two negative cultures and 21 patients who had two positive cultures. There were 5 patients with non-concordant cultures as either their first (n=2) or their last (n=3) culture was negative for *P. aeruginosa*. Of the patients with two *P. aeruginosa* positive cultures (n=21), 17 patients (81%) had complete concordance of isolated strains belonging to the same MT or CC for both cultures.

patient ID	2001			2010	
				C1	C2
1	27			27	27
				9	
2	341			27	
4	27			27	
8	338			27	27
9	NEG			2	
11	27			27	27
13	27			27	27
16	27			27	27
18	27			27	27
20	27	13		385	385
27	27			27	27
30	27			27	27
31	225	27		27	27
				225	
39	27			27	27
40	27			27	
41	27			27	
				230	
42	27			27	176
				176	
43	98			98	
47	NEG			27	27
48	27	346	343	11	
				346	
50	27			27	
				11	
58	9			27	27
60	27			27	27
62	13			27	
63	11			345	345
				21	
64	27			11	
65	9			9	9
66	11	315		9	9
68	44	11		44	44
69	300			300	300
70	9	388		388	
				11	
77	11	337		11	
78	44			44	44
80	9			9	
81	11			300	
82	11			11	
				360	
83	24			11	

Figure 3 Overview of singleton MLVA types (MTs) or clonal complexes (CCs) found per patient during the summer camp study and 2010 (c1= culture 1 and c2= culture 2). Dark red squares represent the highly prevalent clone, CC27. Light red squares represent other shared clones (found in at least two patients).

PERSISTENCE OF CLONAL COMPLEXES

In 2001, MTS belonging to a total of 15 different singleton MTS or CCs were found in 41 participants, and in 2010 17 different singletons or CCs were detected. Table 1 shows the prevalence of the detected clonal complexes and singleton MTS. CC27 is by far the most prevalent clonal complex in both 2001 and 2010 (found in 18 and 20 patients respectively), followed by CC11 (found in 6 and 7 patients respectively). Table 1 also shows the persistence per CC/singleton, where persistence was defined as present in 2001 and in 2010 in the same patient. When comparing persistence between CC27 (persistent in 83%) and other singletons or CCs (mean persistence 33% for CC11 and 48% for all other MTS/CCs), CC27 was significantly more persistent (Figure 4).

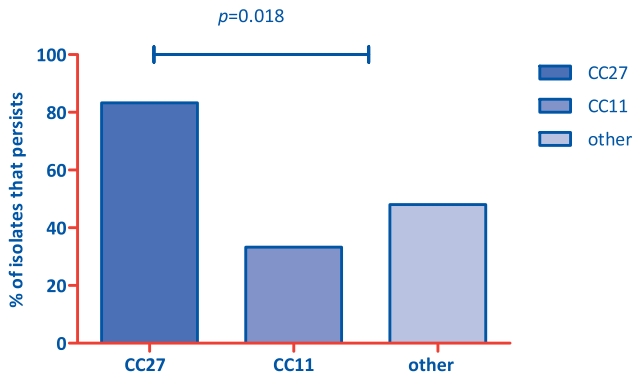

Figure 4 Prevalence and persistence of clonal complexes (CCs)/MLVA types (MTS)

CLINICAL CHARACTERISTICS

Table 2 displays the baseline characteristics of the study participants. Although patients with CC27 in 2001 were significantly younger than patients harboring sporadic clones, there was no significant difference in clinical parameters like pulmonary function or BMI. A trend could be observed of increased treatment requirements for patients with sporadic clones, as these patients were more likely to use inhaled antibiotics or to have required intravenous antibiotics in 2001.

Table 1 Clonal complexes or singleton (non-related) MTS and persistence within patients

clonal complex or singleton MT	2001		2010		persistence in patient	
	frequency	percent	frequency	percent	frequency	percent
2	0		1	2,1		
9	5	11,6	4	8,3	3	60,0
11	6	14,0	7	14,6	2	33,3
13	2	4,7	0	0,0	0	0,0
21	0	0,0	1	2,1		
24	1	2,3	0	0,0	0	0,0
27	18	41,9	20	41,7	15	83,3
44	2	4,7	2	4,2	2	100,0
98	1	2,3	1	2,1	1	100,0
176	0	0,0	1	2,1		
225	1	2,3	1	2,1	1	100,0
295	0	0,0	1	2,1		
300	1	2,3	2	4,2	1	100,0
337	1	2,3	0	0,0	0	0,0
338	1	2,3	0	0,0	0	0,0
341	1	2,3	0	0,0	0	0,0
343	1	2,3	0	0,0	0	0,0
345	0	0,0	1	2,1		
346	1	2,3	1	2,1	1	100,0
360	0	0,0	2	4,2		
385	0	0,0	1	2,1		
388	1	2,3	1	2,1	1	100,0
391	0	0,0	1	2,1		

Table 2 Baseline characteristics of participants.
[a]t-test [b]Chi-square [c]Fischer's exact [d]Mann-Whitney U

	sporadic clone (n=17)	MT27 (n=18)	*p*
age, mean	16.6 (SD 3.8)	12.9 (SD 2.5)	0.002[a]
male, n	9 (52.9%)	9 (50.0%)	0.86[b]
dF508 homozygosity, n	10 (58.8%)	8 (61.5%)	0.67[c]
FEV_1 % of predicted, mean	75.0 (SD 28.3)	86.3 (SD 23.8)	0.25[a]
FVC % of predicted, mean	88.0 (18.1)	91.1 (19.3)	0.65[a]
previous camps visited, mean	6 (SD 3.5)	5 (SD 2.7)	0.51[a]
use of inhaled antibiotics, n	11 (65%)	4 (22%)	0.03[b]
use of rhDNase, n	12 (70.6%)	8 (47.1%)	0.16[b]
IV antibiotics courses in 2001, median	1.4 (IQR 0-2)	0.92 (IQR 0-1)	0.16[d]
CFRD, n	3 (17.6%)	2 (12.5%)	1.0[c]

A survival analysis was performed to study whether patients who harbored 27 in 2001 had an increased risk of lung transplant or death (Figure 5 shows the survival curve). The total at risk time was 3016 months for patients who did not have CC27 in 2001, and 4467 months for patients who did have CC27 in 2001. During at risk time there were 25 events (lung transplantation 12 and death 13). The unadjusted hazard ratio from the Cox proportional hazards model was 0.72 (95% CI 0.33 – 1.58) for patients with CC27 in 2001 (Table 3). In the multiple model, adjustments were made for age and sex. Adjusted hazard ratios showed that CC27 was not significantly associated with lung transplantation or death (HR 1.01, 95% CI 0.41 – 2.49), whereas age did show a trend towards an association (HR 1.01 per month, 95% CI 1.00 – 1.02).

Lung function measurements were collected between 2001 and 2010 and if more than 1 measurement per three months was available, the highest FEV_1 % of predicted was included in the analyses. One patient was excluded from this analysis because of missing lung function data. For the analysis, patients were divided into two groups: patients who were persistently infected

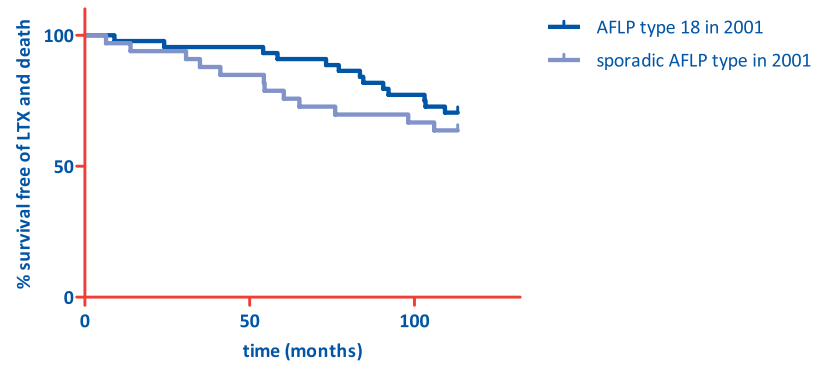

Figure 5 Survival free of lung transplantation or death

with CC27 (n=14), and patients who were not persistently infected with CC27 (n=20). A total of 845 lung function measurements were included (mean 25 measurements per patient). Figure 6 shows the FEV_1% of predicted values by time since the camp. Patients who did not have persistent CC27 infection had a significantly lower estimate of lung function throughout the study period (-20.1, 95% CI -40.1 – -0.17, p=0.048). However, when adding an interaction term with time to the model (time*persistent CC27) this did not significantly improve the model (p=0.79), indicating that there was no significant difference in decline of FEV_1 between the groups.

Table 3 Unadjusted and adjusted hazard ratios for lung transplantation or death

	HR (95% CI)	HR (95% CI)
AFLP type 18 in 2001	0.72 (0.33 - 1.58)	1.01 (0.41 - 2.49)
male		0.50 (0.21 - 1.17)
age at camp		1.01 (1.00 - 1.02)

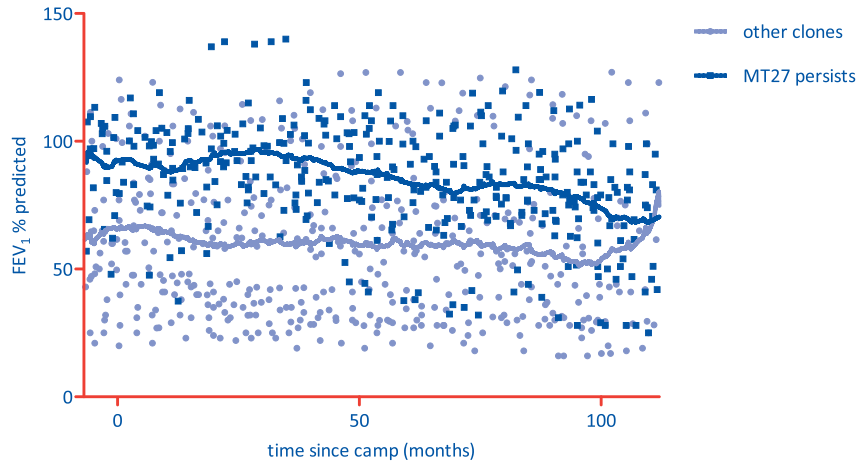

Figure 6 Lung function over time in relation to persistence of CC27. Graph represents the highest FEV_1% of predicted values per three months, for each patient (845 measurements in total). Trend lines (Lowess curves) are shown.

DISCUSSION

In the present study we show that a clone that is highly prevalent in the Dutch CF population displays increased persistence over a nine-year period. This implicates an important role for increased persistence in the success of CC27. Besides increased persistence the clone was not associated with an increased risk of lung transplantation or death. In patients who were persistently infected with CC27 lung function decline was not significantly different from that in patients infected with other highly prevalent clones. This suggests that although CC27 does not appear to be a highly virulent clone, compared to sporadic clones it is a more successful colonizer of the lungs, being able to persist for prolonged periods.

Although we have shown an important role for persistence in the success of CC27, we can only speculate as to the role of transmission in this regard. The 2001 study did show that several cases of probable transmission involved AFLP type 18, but many of the patients with type 18 were already infected upon entering the camp, suggesting that it was already present before the camp[13]. Indeed transmission during a previous camp is plausible (patients were treated

in different centers all over the country so transmission in a CF center is less likely), but another possibility is the presence of an environmental source e.g. a tap, shower or other water source from which several patients could have acquired the same clone. Furthermore, the widespread presence of certain *P. aeruginosa* clones in the environment has been suggested to contribute to the high prevalence of clones[3]. However, the Dutch highly prevalent clone could not be found upon analysis of *P. aeruginosa* isolates from non-CF patients from different wards and out-patient clinics in the hospital and patients on ventilation in the ICU, or patients with otitis media or urinary tract infections, which would suggest that a widespread prevalence of this clone in the environment is less likely[17;19].

A possible explanation for the finding that CC27 is not associated with an increased risk of lung transplant or death could be that CC27 represents a clone that is not characterized by increased virulence. Jelsbak et al. also showed that a highly prevalent *P. aeruginosa* clone in Copenhagen CF patients displayed decreased virulence in a Drosophila model[20]. The increased persistence further suggests that although its virulence is probably decreased, CC27 is possibly better adapted to maintain in the CF lung. This would be in concordance with observations by Bragonzi et al., who show that CF isolates can show attenuated acute virulence, while still maintaining the capacity of causing chronic infection[21]. It can be speculated that the attenuated virulence in CC27 strains results in a less outspoken host immune response towards the pathogen, hereby contributing to its increased persistence.

One of the strengths of this study is the follow-up time of nine years which allowed for a longitudinal analysis of lung transplantation and death risk. Also, the initial evaluation of the *P. aeruginosa* genotype was performed without the knowledge of the occurrence of a highly prevalent clone as the 2001 study was performed with the intention of investigating *P. aeruginosa* transmission between patients. Many studies that investigate highly prevalent *P. aeruginosa* clones in CF are prompted by the occurrence of such clones and are prone to ascertainment bias, since in those cases where transmission within CF centers was observed, the detection of highly prevalent clones is more likely in patients who are more severely diseased, and visit the hospital more often[22]. In addition, the use of MLVA for typing has several advantages over Pulsed Field Gel Electrophoresis (PFGE, a commonly used method in typing studies of CF isolates). MLVA offers increased typeability, ease of interpretation and the possibility of comparison with international typing data and a high discriminatory

index[17]. In this study the data were analyzed on the level of clonal complexes. Because of the high discriminatory index of MLVA and because most of the single locus variants were found to co-exist in the lung of one patient at the same time (58%), SLVs seem to represent a diversity of one clone rather than completely different genotypes. The existence of more SLVs within the patient could represent in-patient evolution in which slightly different variations of one clone adapt to the different niches in the lung, e.g. more and less oxygenized or different levels in the biofilm.

A limitation to our study is the participation of 62% of all eligible patients, which could have led to a selection bias. If the distribution of the highly prevalent clone and the severity of lung disease would have been different in non-participants this could have influenced our results. However, upon studying the distribution of CC27 (AFLP type 18) in the participants and non-participants, there was no significant difference (in 2001 AFLP type 18 was found in 47 (59%) out of all 80 participants and in 18 (44%) out of the 41 participants in the present study, p=0.12). This indicates that the distribution of AFLP type 18 in the group of participants is a reasonable representation of its distribution in the original group of participants. Also, the high concordance between the two samples (for the 30 patients who sent two samples) indicates that one sample offers a sufficiently good representation of the genotypes present in the patient's airways.

Since segregation measures were already implemented in the Netherlands before the detection of a highly prevalent clone, it is important to study its clinical impact. The here presented observations that this clone is not associated with adverse clinical outcome, as was suggested from cross-sectional data, should be taken into account when evaluating the current infection control policies in Dutch CF centers. At least further prospective studying, in a large CF population, of the longitudinal effects of this clone is warranted. Possibly, future results could lead to a revision of the segregation policy in the Netherlands.

CONCLUSION

A *P. aeruginosa* clone highly prevalent in the Dutch CF population showed increased persistence compared to sporadic clones over a period of nine years, while not being associated with an increased risk of lung transplant or death.

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Chapter 8

LONGITUDINAL FOLLOW-UP OF DUTCH CYSTIC FIBROSIS PATIENTS INFECTED WITH A HIGHLY PREVALENT PSEUDOMONAS AERUGINOSA CLONE

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MANUSCRIPT IN PREPARATION.

Abstract

Background

In recent years, several reports have been published on highly prevalent Pseudomonas aeruginosa clones in cystic fibrosis patients. Some clones were suggested to be associated with adverse clinical outcome, but recent longitudinal studies have shown conflicting results. Infection control policies have been implemented to limit the spread of such clones. A recent cross-sectional study showed no association between a newly discovered clone in the Dutch CF population (ST 406) and adverse outcome. We aimed to verify these observations by studying the association between ST406 infection and clinical outcome in a longitudinal follow-up study.

Methods

A longitudinal cohort study was performed in 219 patients diagnosed with a highly prevalent P. aeruginosa clone in The Netherlands in 2007. The clone (ST406) was diagnosed using Multiple Locus Sequence Typing (MLST), in the two largest CF-Centers of the country. Of these patients, clinical data were recorded from the center's databases from 2007 through 2010. A linear mixed model analysis was performed to analyze differences in lung function and lung function decline. A survival analysis was performed using a Cox proportional hazards analysis.

Results

Of 219 patients, 40 (18%) had ST406. Patients with ST406 were significantly younger and more likely to use inhaled antibiotics compared to other patients. Longitudinal lung function measurements were available for 201 (92%) patients. There was no significant difference in lung function decline between patients with ST406 and patients with other P. aeruginosa clones throughout the follow-up period (p=0.35). After adjusting for age, a Cox proportional hazard analysis showed no significant difference in HR of death or lung transplantation for ST406 after adjusting for age (HR 2.41, 95% CI 0.85-6.88, p=0.10). However, ST406 patients were more likely to use inhaled antibiotics throughout the study period (p=0.036).

Conclusions

Despite its high prevalence in the Dutch CF population, the P. aeruginosa clone ST406 is not associated with a more severe lung function decline or increased risk of death or lung transplantation over a three-year follow-up period. There was however an increased use of inhaled antibiotics, indicating the need of further continued monitoring to study the clinical effects in the long term.

Introduction

Pseudomonas aeruginosa is a ubiquitous pathogen that mostly resides in aqueous environments. About 60-80% of patients with CF become chronically infected with *P. aeruginosa*. Although many patients acquire *P. aeruginosa* from the environment[1-3], the occurrence of several highly prevalent clones has been reported from CF centers around the world, suggesting a role for patient to patient transmission. The first studies on these clones reported an association with adverse clinical outcome[4-7], and these findings lead to the implementation of infection control policies (including cohort segregation) to limit the spread of these clones[8]. However, most of the early reports on these clones were cross-sectional and many of these investigations were prompted by the discovery of a *P. aeruginosa* clone with a specific antibiotic resistance profile, precluding firm conclusions about the effect of such clones on CF lung disease. Recent longitudinal studies showed conflicting results regarding the association between infection with a widespread clone from the UK and adverse clinical outcome[9;10].

In the Netherlands, infection control policies to limit patient to patient spread of *P. aeruginosa* were implemented in 2005 and included individual segregation of patients, both in the inpatient and outpatient setting, and advising against physical contact[11]. In 2007, a large study on the population structure of *P. aeruginosa* was performed in the population of two large Dutch CF centers. This study used Multi Locus Sequence Typing (MLST) and revealed the presence of a highly prevalent clone designated ST406[12]. This clone was not directly genetically related to other international clones, was exclusively found in CF patients[13], and upon cross-sectional analysis, it was not associated with adverse clinical outcome[14]. These results suggest that infection with ST406 does not lead to unfavorable outcome, but this needs to be verified in a longitudinal study. We hypothesized that ST406 represents a clone that is not associated with a more severe decline in lung function or decreased survival over a three-year follow-up period. We aimed to study this by performing a longitudinal follow-up study in the cohort of patients investigated in the cross-sectional study of 2007.

METHODS

PATIENTS AND CLINICAL DATA COLLECTION

CF patients under treatment of the Utrecht and the The Hague CF center and who were chronically infected with *P. aeruginosa* were included in the analysis in 2007. Of these patients, those who had a history of lung transplantation or

B. cepacia colonization were not included and patients with available MLST data were included in the longitudinal analysis. Of the participants, demographics and clinical data on pulmonary function and comorbidities were collected from the CF database of each center. The use of inhaled antibiotics was assessed per year. To account for differences in frequency of pulmonary function measurement, only the highest Forced Expiratory Volume in one second (FEV_1) per three months was included. Lung function values were converted into percent of predicted values for FEV_1 based on reference values for either adults[15] or children[16] where appropriate (using only one set of reference values per patient, i.e. the reference set that was appropriate for the majority of measurements). Patients were grouped according to *P. aeruginosa* sequence type (ST) as determined in 2007[12]. Those who had ST406, regardless of the presence of other ST's, were included in the ST406 group. Patients with other clones were assigned to the 'other clones' group. Patients gave written and informed consent to store their data in a database for scientific purposes and the use of this database is permitted by the institutional review board.

STATISTICS

Normality of data was tested using the Kolmogorov-Smirnoff test and for normally distributed data, the Student's t-test was performed to study significant differences. For non-normally distributed data the Chi-square test or Fisher's exact test were used where appropriate. A Cox proportional hazards model was performed to study the association between ST406 and time to death or lung transplantation (combined endpoint). In case of death following lung transplantation, the date of lung transplantation was designated time of censoring.

For longitudinal analyses of lung function, a linear mixed model was used. The model assumed a linear trend in FEV_1 over time for each patient, and allowed for random patient-specific slope and intercept. Several possible confounding factors were added to the model and tested for significance. A difference in decline of FEV_1 between the two groups (ST406 or other clones) was tested by examining the improvement in model fit after adding an interaction term to the model (time*ST group). This interaction term would allow for different slopes over time for the two groups. All analyses were performed using SPSS for Windows, version 15.0.

RESULTS

PATIENTS

In total 219 patients were included in the analysis (Figure 1) with MLST type available. Of these patients, 132 (60%) were from the Utrecht CF center and 87 (40%) were from the CF center in The Hague. In total, forty patients (19%) were infected with ST406 in 2007, and 179 patients were infected with other clones. There were several differences in baseline variables between patients who had ST406 in 2007 and those with other clones (Table 1). Age was significantly lower for patients with ST406 when compared to those with sporadic clones (18.9 and 25.0 respectively, p=0.005). Also, patients with ST406 were more likely to use inhaled antibiotics than those with sporadic clones (80% and 64% respectively, p=0.048). Over the total study period the median proportion of time on inhaled antibiotics use was 1.0 (IQR 1.0 – 1.0) for ST406 patients and 1.0 (IQR 0.25-1.0) for patients with other clones, p=0.036.

LONGITUDINAL LUNG FUNCTION ANALYSIS

Lung function measurements were available for 201 (92%) patients. Eighteen patients were not included in the analysis because they were too young for pulmonary function testing (n=7), moved to another center or were irregular visitors (n=4) or had no lung functions available after the typed culture because of lung transplantation or death shortly after the genotyped culture (n=7). In total 1903 lung function measurements were collected (mean 9.5 measurements per patient). The course of pulmonary function per ST group (means of FEV_1 percent of predicted for patients with ST406 and patients with other clones) is depicted in Figure 2. To analyse the difference in pulmonary function decline, a linear mixed model analysis was performed. The simple model included time and ST group (ST406 or other clones) (Table 2). ST406 patients did not have a significantly different FEV_1 throughout the study period (estimate 3.60, 95%CI -4.35 - 11.54, p=0.37), and adding an interaction term with time to the simple model did not significantly improve it (p=0.33). To this model, age and the use of inhaled antibiotics were added to create a multiple model, as these factors were significantly different at baseline. Adding an interaction term between time and ST group did not significantly improve this model (p=0.35), indicating that lung function decline in the two groups was not significantly different between ST groups. In order to investigate the effect of inhaled antibiotics on the course of lung function, an interaction term between inhaled antibiotics use and time was added to the model.

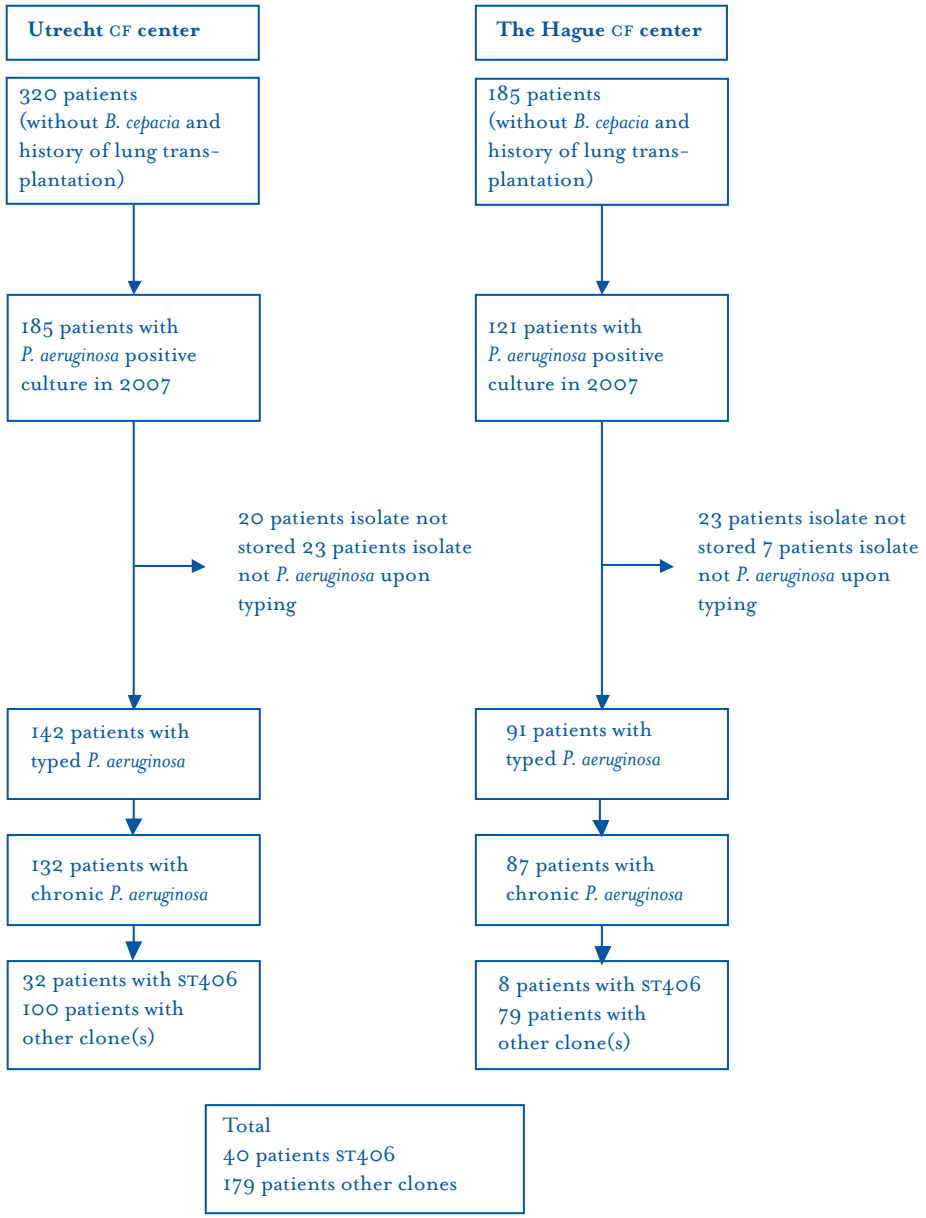

Figure 1 Flow-chart of patient selection

This did not significantly improve the model (p=0.76), suggesting that the use of inhaled antibiotics did not influence the course of lung function over this three-year follow-up period.

Table 1 Baseline characteristics of patients [a]t-test [b]Chi² [c]Mann Whitney U

	ST 406	other clones	p
age, mean (SD)	18.9 (3.7)	25.0 (13.5)	0.005[a]
sex, n (%)	20 (50)	91 (51)	0.92[b]
homozygosity dF508, n (%)	30 (75)	98 (61)	0.10[b]
ABPA, n (%)	3 (7.5)	34 (19)	0.08[b]
CFRD, n (%)	9 (23)	40 (22)	0.98[b]
hospitalizations, median (IQR)	0 (0-1)	0 (0-1)	0.20[c]
FEV^1, mean (SD)	67.3 (27.3)	61.9 (23.5)	0.35[a]
BMI z-score, mean (SD)	-0.5 (1.2)	-0.4 (1.1)	0.53[a]
pulmozyme, n (%)	18 (45)	86 (48)	0.73[b]
inhaled antibiotics, n (%)	32 (80)	114 (64)	0.048[b]
age at diagnosis, median (IQR)	0 (0-3)	0 (0-4)	0.25[c]

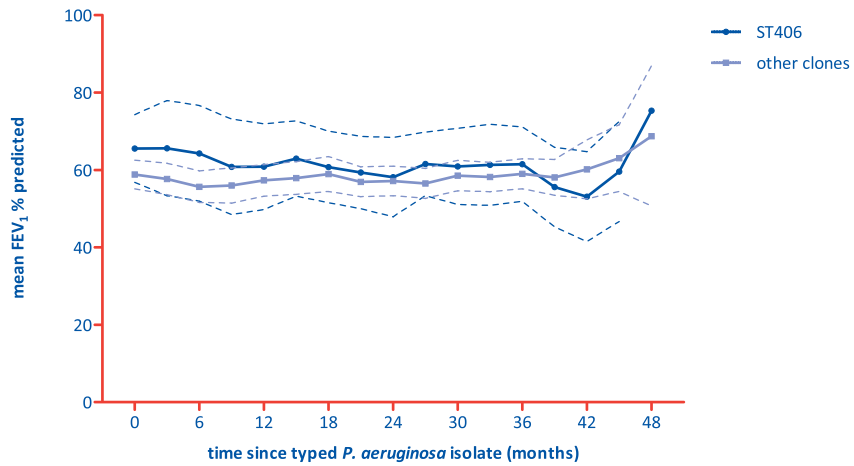

Figure 2 Mean FEV_1 % of predicted during longitudinal follow-up. Dotted lines represent 95% CI's

Table 2 Estimates of FEV_1 percent of predicted based on mixed model analysis

	simple		multiple	
variable	estimate	95% CI	estimate	95% CI
intercept	59.17	55.75 – 62.58	85.32	77.45 – 93.20
ST406	3.60	-4.35 – 11.54	-0.43	-7.84 – 6.98
slope (ST406*time)	-0.59	-1.80 – 0.61	-0.57	-1.77 – 0.63
using inhaled antibiotics			-10.05	-15.94 – -4.16
time (in years)	-1.19	-1.71 – -0.67	-0.45	-1.02 – 0.12
age (in years)			-0.75	-1.00 – -0.51

SURVIVAL ANALYSIS

For the survival analysis, all 219 patients were included. The total follow-up time was 693.3 patient years. Fourteen patients died and eleven patients received a lung transplant. The relative risk (RR) for death or lung transplantation was 1.41 for those infected with ST406 (95% CI 0.60 – 3.31, p=0.43). Figure 3 displays the proportion of patients surviving free of death or lung transplantation over time. In the Cox proportional hazard analysis, the hazard ratio (HR) was not significantly different for ST406 in the unadjusted analysis (HR 1.35, 95% CI 0.54-3.38, p=0.53). The HR changed after adjusting for age, but was not significantly different (HR 2.41, 95% CI 0.85-6.88, p=0.10).

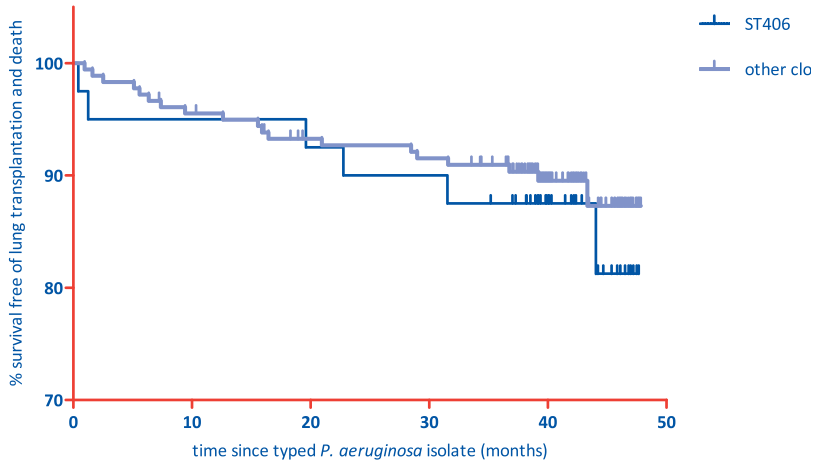

Figure 3 Survival free of lung transplantation and death

DISCUSSION

In the present study we longitudinally analysed the clinical characteristics of patients who were infected with a highly prevalent clone in 2007 and compared these to those with other clones. By showing that there is no difference in the decline of pulmonary function or in survival over a three-year follow-up period, these results further suggest that infection with the Dutch highly prevalent clone is not associated with adverse clinical outcome. Although the use of inhaled antibiotics was higher in patients with ST406, this could not explain the lack of a difference in lung function decline in these patients.

Recent longitudinal studies on the effects of highly prevalent clones in CF patients have presented conflicting results. After initial reports of the detrimental effects of highly prevalent clones from the UK, Jones et al. investigated the longitudinal effects of infection with this two of such clones in 80 CF patients[9]. They found no difference in survival, lung function and nutritional status. However, in this study only a small group of patients were included based on whether they were admitted for inpatient treatment during the second half of 1999. In the present study, we included all patients with a *P. aeruginosa* positive and typed culture in 2007, which means that a large proportion of the total CF population in the center is included, which makes the results more representative for the entire population. Similar to our results, this study showed an increased treatment burden for patients with the highly prevalent clone. The use of inhaled antibiotics was higher in ST406 patients. If the use of inhaled antibiotics slowed lung function decline over a three-year period, this could explain why patients infected with ST406 did not have a stronger decline of pulmonary function. Since the use of inhaled antibiotics did not significantly influence the course of lung function over a three-year period, this was not the case. Another recent study included 446 CF patients, of whom 102 were infected with a highly prevalent clone (either strain A, identified as the Liverpool Epidemic Strain, or a new highly prevalent strain, designated strain B). Similar to the present study, the authors did not find a difference in lung function decline, and there was no difference in treatment burden. There was an increased risk of death or lung transplantation, but only for strain A. Together with our results, it can be concluded that, unlike what has been suggested from previous reports, not all highly prevalent clones are associated with more severe lung disease. Furthermore, although in both recent longitudinal studies some adverse characteristics were found associated with certain highly prevalent clones, it is difficult to attribute these differences to a causal effect of

the highly prevalent strain, since the majority of patients were already infected with *P. aeruginosa* upon inclusion into the study. It is possible that these particular clones infect more severely diseased lungs (either because of more frequent patient-to-patient contact or because of a better ability to adapt to that specific lung environment) and represent markers of severe lung disease.

There are several limitations to this study. Firstly, the presence of the clone was assessed at the start of the observation period only. Therefore it is unknown which clones patients were infected with before 2007 and for how long, as is the case for many of the other studies on highly prevalent clones. More importantly, it is unknown whether patients were still infected with the same *P. aeruginosa* genotype in 2010 as in 2007. A change from infection with the ST406 clone to infection with another clone after 2007 is less likely, since in a recent study in which 41 patients were followed for a period of 10 years, we show that persistence of the ST406 clone (persistent in 83% of patients initially infected) was significantly higher when compared to other clones (persistent in 48%) (de Vrankrijker et al., submitted). A change from a non-ST406 clone to ST406 by patient-to-patient transmission also seems less probable. The segregation policy was implemented in 2005, and this included the discontinuation of summer camps for CF patients and individual segregation of patients in the CF center. Another possible limitation is the lack of lung function measurements for seven patients who died or had a lung transplantation shortly after the genotyped culture. If these severely diseased patients were all infected with ST406, this could have biased the results. However, only two of these patients were indeed infected with ST406, making it less likely that this influenced the lung function analysis.

After the first reports on the detrimental effects of highly prevalent *P. aeruginosa* clones in the Netherlands, infection control policies in the Netherlands were more strict than in most other countries[11]. With the present study, we show that the highly prevalent clone in the Dutch CF population is not associated with unfavorable clinical outcome. This suggests that high prevalence of certain *P. aeruginosa* clones does not necessarily lead to more severe lung disease. However, there was an increased use of inhaled antibiotics in patients with ST406. In our study the treating physicians were unaware of the *P. aeruginosa* genotype of their patient, as opposed to several other studies. Thus the increased use of antibiotics might indicate an increased need for treatment in ST406 patients, while other clinical parameters are not (yet) different. Since our data represent the first results after implementation of the infection control policies, it is important to continue monitoring the occurrence of highly prevalent *P. aeruginosa* clones and the associated clinical characteristics over a prolonged period of time. When the effects of such clones in the long term are still not different from other clones, a revision of the current infection control policies might be considered in the future, for instance the introduction of cohort segregation like in certain other countries[8]. Additional research on the effect of the current infection control policy on the *P. aeruginosa* population structure in the Dutch CF population is currently ongoing and will offer more insight into the efficacy of individual segregation.

CONCLUSION

The highly prevalent clone ST406 is not associated with more severe lung function decline or increased risk of death or lung transplantation over a three year period, suggesting that high prevalence of *P. aeruginosa* clones is not necessarily associated with adverse clinical outcome. An increased use of inhaled antibiotics in ST406 patients however does warrant continued monitoring of the clinical impact of this clone.

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Chapter 9

USE OF INHALED TOBRAMYCIN AND RISK OF AMINOGLYCOSIDE RESISTANCE IN PSEUDOMONAS AERUGINOSA FROM CYSTIC FIBROSIS PATIENTS

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SUBMITTED

Abstract

Background

The use of inhaled tobramycin has become the mainstay of treatment in cystic fibrosis (CF) patients chronically infected with Pseudomonas aeruginosa. The follow-up time of tobramycin inhalation trials is limited and thus the effect of inhaled tobramycin on aminoglycoside resistance after long-term treatment is not clear. We aimed to study the effect of tobramycin inhalation on aminoglycoside resistant P. aeruginosa in a cohort of CF patients who were followed for up to eight years.

Methods

A retrospective cohort study including patients from the Utrecht CF center was performed between 2002 and 2009. Patients were included if they had P. aeruginosa positive sputum cultures during the observation period and if they started inhaled tobramycin after the first P. aeruginosa positive culture. A Cox proportional hazards model was used to study whether tobramycin inhalation was an independent risk factor for the development of aminoglycoside resistant P. aeruginosa. A linear mixed model was used to study the difference in lung function decline before and after occurrence of resistance.

Results

In total, 210 patients met the inclusion criteria. Aminoglycoside resistant P. aeruginosa was cultured in 50 (24%) patients. In the Cox proportional hazards analysis tobramycin inhalation showed an independent association with aminoglycoside resistance (adjusted HR 3.38 (95% CI 1.78 – 6.16)). There was no significant difference in lung function decline before and after the resistant P. aeruginosa was cultured.

Conclusion

The long-term use of inhaled tobramycin in CF patients is an independent risk factor for aminoglycoside resistant P. aeruginosa.

Introduction

Cystic fibrosis (CF) lung disease is characterized mainly by chronic inflammation and infection, leading to progressive obstruction and a subsequent decline in lung function. The most important pathogen in CF lung disease is *Pseudomonas aeruginosa*, a ubiquitous gram-negative bacterium. Patients acquire *P. aeruginosa* at a young age, and by the time they reach adulthood 60-80% of CF patients are chronically infected[1]. Once the infection has become chronic, *P. aeruginosa* is rarely eradicated, and chronic infection is an important factor in morbidity and mortality in these patients[2].

The most important aminoglycoside for treating pulmonary infections in CF is tobramycin, which is frequently administered intravenously (IV) for the treatment of acute exacerbations. The penetration of tobramycin in sputum upon IV administration is poor, which is why high dosages are necessary to achieve sufficient concentrations for the inhibition of *P. aeruginosa*[3] High doses of IV aminoglycosides however are associated with systemic side effects, like nephrotoxicity and ototoxicity. In the 1990's, tobramycin for inhalation became commercially available for use in CF patients. The advantage of administering antibiotics per inhalation is that by direct delivery to the endobronchial site of infection high concentrations of antibiotic are achieved in the sputum[4]. Because systemic absorption is limited, the risk of systemic side effects is minimal. Trials investigating the efficacy of tobramycin inhalation in CF patients showed that tobramycin inhalation not only decreased the density of *P. aeruginosa* in sputum, but also decreased hospitalizations and led to improvements in pulmonary function[5–7]. Due to these successful results, the use of inhaled tobramycin has become important in the maintenance treatment of CF patients chronically infected with *P. aeruginosa*[8].

Upon treatment of patients with inhaled antibiotics, significant decreases in bacterial loads are observed. However despite the constant pressure of antibiotics, *P. aeruginosa* is rarely eradicated completely[9], which raises concerns about the development of antibiotic resistance. In trials of tobramycin inhalation efficacy and safety, increases in MIC values have been observed[6,7,10,11], although after 6 months there was no indication of selection for the most resistant isolates to become the most prevalent[10]. These trials had follow-up times of 96 weeks or less, but with the increasing life span of CF patients, many CF patients are treated with inhaled tobramycin for several years. Therefore,

the long-term effect of inhaled tobramycin on aminoglycoside resistance is not yet clear. We hypothesized that upon long-term follow-up, the use of inhaled tobramycin is independently associated with aminoglycoside resistance. In the present study we aimed to study the effect of tobramycin inhalation therapy on the development of aminoglycoside resistance in *P. aeruginosa* in a cohort of CF patients who were followed for a period of up to 8 years. In addition, we aimed to study the change in lung function decline before and after the occurrence of aminoglycoside resistant *P. aeruginosa*.

METHODS

PATIENTS AND STUDY DESIGN

We performed a cohort study of patients who regularly visited the Utrecht CF Center from January 1, 2002 through December 31, 2009. This center currently manages multidisciplinary CF care for approximately 380 patients, both adults and children. Patients were eligible if the first documented *P. aeruginosa* positive culture within the observation period was aminoglycoside sensitive and if they did not have a history of an aminoglycoside resistant *P. aeruginosa*. Furthermore, patients were only included if they started using tobramycin inhalation after the first *P. aeruginosa* positive culture in the observation period (as opposed to patients who were already using tobramycin inhalation before the first *P. aeruginosa* in the observation period).

The results of cough swab and sputum cultures and the associated antibiotic resistance profiles were recorded. An aminoglycoside resistant *P. aeruginosa* was defined as *P. aeruginosa* with intermediate susceptibility or resistance to both tobramycin and amikacin based on results from agar diffusion tests. For classification into resistant or intermediate susceptibility Clinical and Laboratory Standards Institute (CLSI) breakpoints were used[12]: susceptible ≤ 4 µg/ml, intermediate 8 µg/ml, resistant ≥ 16 µg/ml.

Data regarding clinical characteristics of the patients (including clinical complications like CF-related diabetes (CFRD), CF related liver disease (cirrhosis, CFRLD)[13], allergic bronchopulmonary aspergillosis (ABPA), and CF genotype) were collected from the center's CF database, in which data are recorded from annual multidisciplinary check-ups. Forced Expiratory Volume in 1 second (FEV_1) was measured using a pneumotachograph and converted to percentage of predicted values. For the longitudinal lung function analyses the highest FEV_1 value per three months was included. Body Mass Index (BMI)

Z-scores were calculated using standard Dutch growth charts[14]. For the survival analyses, BMI and pulmonary function were recorded at the start of the at risk time.

Inhaled tobramycin is prescribed on a month-on month-off regimen. In the survival analysis, the months that were recorded as positive for tobramycin inhalation reflected the months during which tobramycin was prescribed, and not the use of tobramycin when considering the month-on month-off regimen, as the exact pattern of tobramycin inhalation was not reliably recorded. The use of tobramycin inhalation was only included if tobramycin was prescribed for maintenance treatment, defined as more than three months of treatment, as opposed to eradication therapy. For the analysis, the use of antibiotics and the presence of an aminoglycoside resistant *P. aeruginosa* were assessed per month. Intravenous antibiotics courses, which normally last no longer than a month, were recorded as present in that month. In cases where the occurrence of aminoglycoside resistance occurred in the same month as an intravenous antibiotics course, IV antibiotics was only recorded as present if the course preceded the event.

Patients gave written and informed consent to store their data in a database for scientific purposes and the use of this database is permitted by the institutional review board.

STATISTICAL ANALYSIS

Patients who did or did not use inhalation tobramycin were compared using a t-test, Mann Whitney or Chi-squared test where appropriate. Normality of data was checked using the Kolmogorov-Smirnov test.

A time-to-event analysis was performed with the occurrence of an aminoglycoside resistant *P. aeruginosa* in patients as the outcome. 'At risk time' was defined as the time in months from the first *P. aeruginosa* positive culture during the observation period until a positive culture for an aminoglycoside resistant *P. aeruginosa* or until the last performed culture during the observation period if resistance did not occur. Data were analyzed using a Cox proportional hazard model. In this model, the use of tobramycin inhalation and IV aminoglycosides were incorporated as time-dependent variables. This means that the status for these variables can vary for each time-unit in the analysis (month). Simple Cox regression models were estimated for the antibiotics (tobramycin, colistin and IV aminoglycosides) and potential confounders (demographics, CF genotype,

CF related diabetes (CFRD), allergic bronchopulmonary aspergillosis (ABPA) CF related liver disease (CFRLD), BMI Z-score and FEV_1) individually. Next a multiple model was estimated including antibiotics and potential confounders that were (borderline) significantly related ($p < 0.20$) to aminoglycoside resistance in the simple models. The Cox proportional hazards models were carried out in SAS version 9.2 using the SAS PHREG procedure.

In order to determine whether a change in lung function decline occurred after the endpoint (aminoglycoside resistant *P. aeruginosa*) a mixed model analysis was performed. The model assumed a linear trend in FEV_1% of predicted over time for each patient, and allowed for random patient-specific slope and intercept. The effect of adding an interaction term with time and whether or not resistance had occurred (time*resistance) was investigated to determine whether a difference in slope existed after the occurrence of resistance. Except for the survival analyses, all analyses were performed using SPSS version 15.0 for Windows.

RESULTS

PATIENTS AND ACQUISITION OF RESISTANT P. AERUGINOSA

In total there were 403 patients who had at least one culture taken at the CF center between 2002 and 2009. Figure 1 shows a flow-chart of patient selection. Of these patients there were 296 who ever had a *P. aeruginosa* positive culture in that period. Eighteen patients with an aminoglycoside resistant *P. aeruginosa* in their first positive culture and 14 patients with a history of aminoglycoside resistant *P. aeruginosa* (isolated before the start of the observation period) were excluded from the analysis. Thirty-seven patients who were already using inhaled tobramycin before the first *P. aeruginosa* positive culture in the observation period were excluded. Another 17 patients were excluded because they participated in placebo-controlled trials on the effect of inhaled antibiotics during the study period. In total there were 210 patients included in the analysis. Of these patients, those who had a lung transplant at some point during the observation period (five) were included in the analysis until the time of lung transplant. Table 1 displays baseline characteristics of the patients included in the analysis, showing that patients who started using tobramycin inhalation sometime during at risk time were more likely to use colistin inhalation, but did not have significantly different lung function values at the start of at risk time.

Aminoglycoside resistant *P. aeruginosa* was cultured in 50 (24%) patients at risk: in 25 (36%) patients using inhaled tobramycin and in 25 (18%) patients not using inhaled tobramycin (RR 2.00, 95% CI 1.25 – 3.21).

Table 1 Baseline characteristics of patients (at start of 'at risk' time), by subsequent use of tobramycin inhalation

characteristic	use of inhaled tobramycin		
	yes	no	*p*
patients (n=210)	70	140	
mean FEV1 % of pred (SD)†	79 (19)	78 (25)	0.93[A]
mean BMI Z-score (SD)	-0.28 (0.95)	-0.27 (1.17)	0.94[A]
median age at start in years (IQR)	12 (6-17)	11 (5-18)	0.73[B]
pancreatic insufficiency, n (%)	61 (87)	116 (83)	0.42[C]
male, n (%)	31 (44)	76 (54)	0.17[C]
homozygosity for DF508*, n (%)	43 (61)	87 (62)	0.97[C]
allergic bronchopulmonary aspergillosis, n (%)	3 (4.3)	11 (7.9)	0.39[C]
CF related diabetes, n (%)	4 (5.7)	8 (5.7)	0.63[C]
CF related liver disease, n (%)	3 (4.3)	11 (7.9)	0.39[C]
use of colistin during at risk time, n (%)	22 (31)	26 (19)	0.04[C]
IV antibiotic course during at risk time, n (%)	32 (46)	47 (34)	0.09[C]

*genotype available for 198 patients

† lung function available for 162 patients, [a]t-test, [b]Mann Whitney test, [c]Chi² test or Fisher's Exact Test

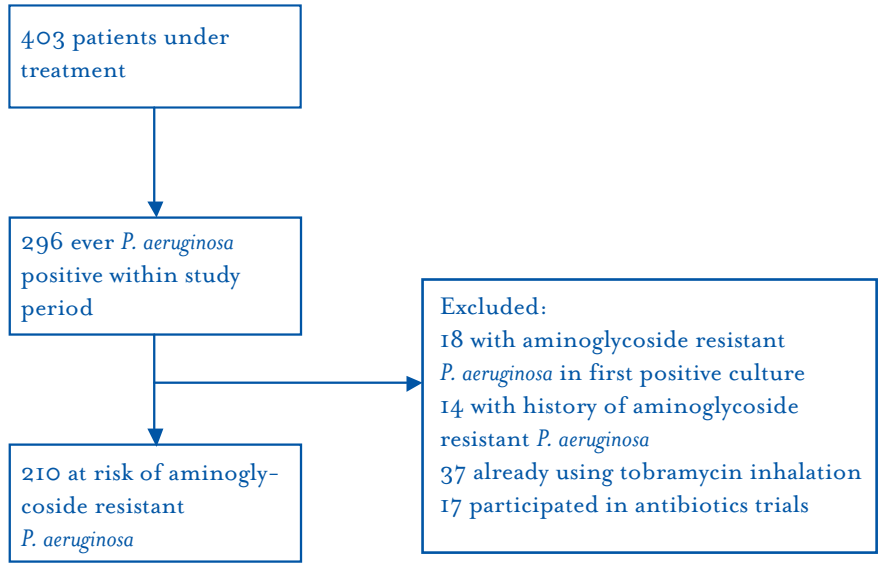

Figure 1 Flow-chart of included patients

RISK FACTORS FOR AMINOGLYCOSIDE RESISTANT P. AERUGINOSA

The total at risk time for the 210 included patients was 10781 months. During at risk time, patients used tobramycin inhalation for 1962 months in total (among the 70 patients who used inhaled tobramycin, the mean time of use within the at risk time was 28 months, SD 17). The total at risk time for patients without aminoglycoside resistance was 8742 months, in which during 1370 months (16% of time) tobramycin inhalation was used. For those with aminoglycoside resistance, at risk time was 2039 months, in which during 529 months (30% of time) tobramycin inhalation was used.

Hazard ratios for the antibiotics investigated based on the simple and multiple models are displayed in Table 2. The unadjusted hazard ratio for aminoglycoside resistance in patients using tobramycin inhalation was 3.38 (95% CI 1.83 – 6.26). The use of IV aminoglycosides (unadjusted HR 4.41, 95% CI 1.04 – 18.73) also showed a significant association with the risk of aminoglycoside resistance. Of the clinical variables analyzed in the univariate analysis only FEV_1 was significantly associated with aminoglycoside resistance (HR 0.97, 95% CI 0.97 – 1.0). BMI Z-score showed a borderline significance (HR 1.32, 95% CI 0.98 – 1.78). In the multiple model, FEV_1 and BMI were therefore included. CFRD was also included since its p-value was close to 0.20

and because of a reported association between CFRD and resistant *P. aeruginosa*[15]. The analyses included only patients who did not have missing data on these variables (47 patients did not have lung function data available; 46 because they were too young to produce reliable lung function test results, and one patient because lung function was not measured around the start of at risk time). Table 2 shows the hazard ratios for inhaled tobramycin and IV aminoglycosides, adjusted for FEV_1, BMI z-score and CFRD. Tobramycin inhalation was still significantly associated with aminoglycoside resistance (HR 3.31, 95% CI 1.78 – 6.16), whereas the association with IV antibiotics became weaker and was no longer significant (HR 3.15, 95% CI 0.73 – 13.69). When the multiple analysis was repeated without inclusion of FEV_1, thereby including all 210 patients, the results did not change significantly.

Table 2 Unadjusted and adjusted hazard ratios for aminoglycoside resistant *P. aeruginosa*

	unadjusted			adjusted*		
	HR	95% CI	*p*	HR	95% CI	*p*
tobramycin inhalation	3.38	1.83 – 6.26	0.0001	3.31	1.78 – 6.16	0.0002
IV aminoglycosides	4.41	1.04 – 18.73	0.04	3.15	0.73 – 13.69	0.13

*In the multiple model, hazard ratio's were adjusted for FEV_1 (Functional Expiratory Volume in 1 second), BMI z-score (Body Mass Index) and CFRD (CF related diabetes).

AMINOGLYCOSIDE RESISTANCE AND LUNG FUNCTION DECLINE

Figure 2 shows the course of pulmonary function of the 50 patients who had aminoglycoside resistant *P. aeruginosa* in relation to time of aminoglycoside resistant *P. aeruginosa* (t=0). The highest FEV_1 per three months was selected for each patient. A total of 769 measurements were included. In order to study whether a significant difference in lung function decline exists between the months before and after aminoglycoside resistance, a linear mixed model analysis was performed. The model did not improve significantly by adding an interaction term with time to the model (p=0.35) implying there was no significant difference in pulmonary function decline before and after resistance.

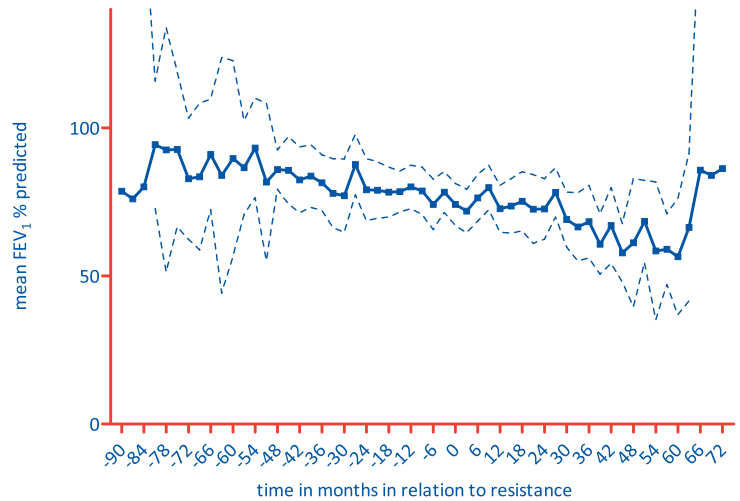

Figure 2 Lung function in relation to time of aminoglycoside resistant *P. aeruginosa*. Graph shows mean FEV_1 per three months in relation to the occurrence of resistant *P. aeruginosa*. For each patient, the highest FEV_1 per three months was included. Dotted lines represent 95% confidence interval.

DISCUSSION

The use of inhaled tobramycin is independently associated with an increased risk of developing aminoglycoside resistant *P. aeruginosa*. In the present study patients were followed for up to eight years, which is substantially longer than previous studies investigating the effect of tobramycin inhalation on resistance. The occurrence of resistance was not associated with a more severe decline in lung function.

Although several trials have investigated the risk of aminoglycoside resistance associated with tobramycin inhalation, their reported effects of tobramycin resistance were not very strong. Ramsey et al. report on the safety of tobramycin inhalation from two large randomized controlled trials, in which patients were followed for 24 weeks. The authors observed a trend toward increases in MIC values of tobramycin in the *P. aeruginosa* isolates obtained from the tobramycin group[7]. In a further analysis of the combined microbiology results from these trials, Burns et al. showed that there were significantly more patients in the treatment group with isolates with a tobramycin MIC ≥16 µg/ml

both 20 and 24 weeks after start of the trial (26% vs. 17% after 20 weeks and 23% vs. 8% after 24 weeks)[10]. However, most patients did not have differences in MIC values at the end of the study period compared to baseline, and there was no selection for the most resistant isolate to become most prevalent. Additional follow-up studies of microbiological data collected until 96 weeks after start of tobramycin therapy showed that the proportion of isolates with MIC values ≥16 µg/ml increased from 5 to 19%[6]. The results presented in our study show that of the patients who use inhaled tobramycin 36% had aminoglycoside resistant *P. aeruginosa* and that from a survival analysis, inhaled tobramycin was an independent risk factor for aminoglycoside resistance. This suggests that the relatively short follow-up time of the above-mentioned studies might have underestimated the effect of the use of inhaled tobramycin over a longer period of time. One study on the incidence and risk factors for multiple antibiotic-resistant *P. aeruginosa* (MARPA), in a large cohort followed for up to 5 years, found an association between 'long-term use of inhaled tobramycin' and MARPA acquisition. However, this study did not specifically look at the relation between tobramycin inhalation and the occurrence of aminoglycoside resistance over time. Also, the main outcome was MARPA as opposed to aminoglycoside resistance, meaning that resistance to meropenem and ciprofloxacin were also taken into account in the definition of resistance. The use of oral antibiotics was not accounted for in the analysis, which makes it difficult to interpret the finding of long term inhaled tobramycin as a risk factor[15].

A strength of the present study is the longer follow-up time, which makes it clinically relevant for the current treatment of CF patients. With increasing longevity in CF patients, a longer follow-up time is necessary to study the effects of inhaled antibiotics, since many patients will be treated for more than 2 years. Furthermore, by performing a survival analysis using a Cox proportional hazards model with time-dependent variables, we were able to specifically study the relationship between the timing of tobramycin use and the occurrence of aminoglycoside resistance. Also, the use of this model allowed for other confounding factors to be taken into account, including the effects of intravenous antibiotics. The fact however, that this was a retrospective cohort study means that in spite of correcting for confounders there could have been residual confounding by other unknown factors that might influence the use of tobramycin inhalation and the risk of aminoglycoside resistance.

In the present study, qualitative interpretive categories to define resistance (intermediate susceptibility or resistance) were based on CLSI break-

points[12]. These breakpoints are established for parenteral therapy and do not apply to CF lung infections when treated with inhaled antibiotics[10]. Data from the tobramycin trials show that in about 95% of patients, sputum concentrations exceeding 25 times the MIC of the *P. aeruginosa* isolates were achieved[16]. Also, there was no relation between tobramycin MIC and lung function change[6], which is similar to our finding that patients were not characterized by an increased lung function decline after acquisition of aminoglycoside *P. aeruginosa*. Because the resistant isolates were no longer available for assessment of exact MIC levels, it is not possible to distinguish between the effect of isolates of MIC's just above or much higher than the established breakpoints. It is possible that in patients with highly resistant isolates, there is a significant effect on lung function decline, if the MIC approaches the sputum concentration of inhaled tobramycin. In the tobramycin trials, it was not possible to establish new breakpoints on the basis of clinical response[10] because of the low number of patients with an MIC ≥16 µg/ml.

Although the breakpoints do not apply to inhaled antibiotics, the presence of resistant *P. aeruginosa* might be of influence on the efficacy of intravenous antibiotics, since the IV administration of antibiotics in a sufficient dose to reach adequate sputum concentrations is limited because of systemic toxicity. One study could not identify a correlation between antibiotic susceptibility of *P. aeruginosa* isolates and response after IV therapy for acute exacerbations[17], but it is difficult to study this when a standardized definition for exacerbations is lacking. The identification of tobramycin inhalation as a significant risk factor for aminoglycoside resistance in the present long-term follow-up study does warrant careful monitoring of resistance in CF patients' isolates. With increasing lifespan it is expected that the use of inhaled tobramycin as maintenance therapy will increase further, and patients will be treated for many years, which could lead to a further increase in resistance. Possible measures to prevent this could include the use of new antibiotics, combination therapy of different antibiotics and more restricted indications for its use.

In conclusion, the results presented here show that inhaled tobramycin is an independent risk factor for the development of aminoglycoside resistance in *P. aeruginosa* in patients who were followed for up to eight years. Although the presence of aminoglycoside resistance was not associated with a more severe lung function decline, the occurrence of resistance in relation to this agent warrants ongoing monitoring of *P. aeruginosa* resistance in CF patients.

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Chapter 10

DISCUSSION AND FUTURE DIRECTIONS

DISCUSSION

Over the past decades an important improvement in the prognosis of patients with cystic fibrosis has been realized by a more timely and aggressive treatment of pulmonary infections. A large number of studies added to the knowledge about the epidemiology, clinical effects and possible interaction of pathogens in CF lung disease. In this thesis, these studies are reviewed and new data is added to this field.

Bacterial pathogens and especially *Pseudomonas aeruginosa* play an important role in CF. Increased microbiological monitoring, improved (culture-independent) detection techniques but also improved (early) antibiotic treatment of classic CF pathogens, possibly creating a niche for new organisms, have led to a changing epidemiology of pathogens (Chapter 2). *P. aeruginosa* remains highly prevalent, and the occurrence of highly prevalent clones in CF populations has added another level of complexity to *P. aeruginosa* epidemiology. Furthermore, besides bacterial pathogens, respiratory viruses and fungi are also found to be of increasing importance (Chapter 2 and 4), either by their individual effect on lung disease, or by a possible interaction with other pathogens. RSV can play a facilitating role in *P. aeruginosa* airway infection (Chapter 3), but it is conceivable that many other pathogens are able to interact to some extent, either by influencing the host or by directly interacting to increase pathogenicity.

For many of the newly detected or 'emerging' pathogens, the clinical significance for CF patients has not yet been elucidated. Although some are associated with adverse clinical outcome upon cross-sectional analysis, for many pathogens, a longitudinal analysis fails to show a significant effect on pulmonary function decline (Chapter 2, 4, 6, 7 and 8). This implies that some pathogens might be merely markers of more severe lung disease. The multifactorial nature of CF lung disease complicates studying the association between individual pathogens and severity of lung disease. The temporal relationship between the detection of pathogens and the progression of lung disease has been not fully elucidated, since it is still not entirely clear whether inflammation precedes bacterial colonization or whether inflammation is a result of chronic colonization in CF. More specifically, although the genotype data on isolated *P. aeruginosa* from Dutch CF patients in this thesis date back to 2001 when at least some of the spread of the highly prevalent clone took place, the lack of prospective data on the introduction of the ST406 clone into the Dutch CF population complicates research on its clinical impact. More generally, in our observational studies, although we evaluated pulmonary function decline over time and corrected for possible confounders, there might be other yet unknown influencing factors that change over time; changes in treatment strategy (including the use of adjuvant agents like mucolytics, anti-inflammatory drugs, but also physiotherapy), patient adherence to therapy and individual differences in host-pathogen interactions might all influence the severity of lung disease, thereby obscuring the effect of the pathogen.

Improved and intensified treatment of CF patients has resulted in a drastic increase in life expectancy. The improved treatment includes increased use of inhalation and oral antibiotics and other adjuvant agents, but also other measures like physiotherapy, nutrition supplements and infection control policies including segregation measures. Despite the beneficial effects of many of these treatment measures, intensified treatment has its drawbacks. The daily intake of pancreatic enzymes, inhaled antibiotics and adjuvant drugs is time-consuming and can be considerably burdensome to patients. The use of maintenance antibiotics has indeed proven to be beneficial to patients with regards to preserving pulmonary function, but the risk of antibiotic resistance is considerable (Chapter 9). It can be expected that antibiotic resistance will further increase as patients live longer and are treated with antibiotics for prolonged periods of time. Moreover, the increased infection control measures that were implemented in the Netherlands in 2005 included individual segregation of patients both in the hospital and outside. These measures and the discontinuation of summer camps specifically, have been difficult for some CF patients to accept, as it means that they can no longer spend time with other CF patients, which many of them consider to be of much value. Many patients still seek ways to meet with other CF patients (Chapter 5).

FUTURE DIRECTIONS

The findings described in this thesis and recent developments in CF research have implications for future research into microbiological diagnostics, treatment of pulmonary infection and infection control.

MICROBIAL DIAGNOSTICS

With the advent of new culture-independent detection techniques, it is becoming increasingly clear that the infection process in the CF airways is polymicrobial. The different microbes in the CF airway are likely to interact and this could mean that the virulence of the individual species might be influ-

enced by other organisms that are not detected by regular culture techniques[1]. Certain pathogens that were previously regarded as avirulent might have altered virulence upon interacting with other pathogens. This could also explain why certain pathogens that do not have a clinically significant effect on a population level might have an effect in certain individual cases. The polymicrobial nature of CF airway infection implies the need for broad microbiological surveillance, aimed at identifying the microbial diversity in CF airways, instead of focusing on individual CF pathogens. Recent studies into the CF airway microbiome suggest an association between the airway microbiome and age, where species diversity decreases with age[2]. Others reported an association with lung function, which could imply that the microbiome has an effect on lung disease[3]. Future research should focus on the relationship between the airway microbiome and lung disease, e.g. by investigating whether there are microbial markers for predicting pulmonary exacerbations, as it is still not entirely clear what triggers exacerbations. It could be hypothesized that a change in microbiome composition might increase the virulence of one or more pathogens, thus causing an exacerbation. Furthermore, a polymicrobial infection could mean that its response to antibiotics is different, as this might be influenced by interaction between species. Thus research is necessary on the effects of species interaction on pathogen response to antibiotics. Also, interactions between the microbiome and the host might differ between patients. When studying the relationship between the microbial interaction and their interaction with the host, this could give new directions for tailor-made treatment strategies. These might include a more precise use of antibiotics aimed at the most virulent pathogens in the infection but also the use of probiotics to influence the microbiome such that virulence is decreased.

TREATMENT OF PULMONARY INFECTION

We have shown that the use of inhaled antibiotics is a risk factor for antibiotic resistance. With a continuing increase in life span, patients will be treated with antibiotics for many years and it can be expected that antibiotic resistance will further increase. This warrants the search for new antibiotics. As mentioned before, the use of probiotics could be considered if more is known about the microbiome and its association with lung disease. Also, the beneficial effects of adjuvant agents like mucolytics, anti-inflammatory agents or new agents (e.g. those aimed at specific bacterial virulence factors like quorum-sensing inhibitors) should be further investigated. Furthermore, the current indications for (maintenance) treatment with antibiotics should be more clearly defined, to prevent the unnecessary use of antibiotics. Intermittent

therapy, as is the case with inhaled tobramycin, could be investigated for other antibiotics and with increased time intervals, to possibly reduce the risk of resistance. Combination therapy of different antibiotics might not only prevent resistance to one agent, but might also result in synergistic effects[4].
Also, a standardised definition of pulmonary exacerbations would be beneficial to forming more specific guidelines for antibiotic use.

Additionally, recent findings that the CF airway infection process should be viewed as a chronic polymicrobial infection, have several implications for studying the effect of antibiotics[5]. Currently, the choice of antibiotics in a pulmonary exacerbation is based on recent culture and antimicrobial susceptibility results. Mostly, the chosen antibiotics are aimed at a single pathogen, whereas in a polymicrobial infection, the susceptibility of other organisms present might also influence the success of antibiotic treatment. Chapter 9 suggests that in vivo susceptibility is different from in vitro susceptibility, but when considering the infection as polymicrobial, it is possible that the presence of other pathogens alters the antibiotic susceptibility of a certain pathogen in vivo. Furthermore, the chronic nature of the infection implies that the aim of the therapy is not eradication of the pathogen but to suppress bacterial loads and inhibit the expression of certain virulence genes. Therefore, using highly sensitive qualitative cultures or other detection methods aimed at detecting a small number of pathogens to evaluate the effect of treatment during pulmonary exacerbations is inadequate. Assessing the bacterial loads and quantifying the expression of certain virulence genes, together with investigating the airway microbiome, might offer new insight into the way in which antibiotics provide clinical benefit in pulmonary exacerbations[5].

INFECTION CONTROL

We showed that although strict infection control policies were implemented in the Netherlands to prevent further transmission within the CF community, colonization with the highly prevalent clone is not characterised by adverse outcome (Chapters 6, 7 and 8) although there was an increased use of inhaled antibiotics in patients infected with ST406 (Chapters 6 and 8). The results from Chapter 7 further suggest that this clone is highly persistent and not easily replaced by other clones. These observations prompt further research into the effects of the current infection control policy. Currently, *P. aeruginosa* isolates of all CF patients in Utrecht and The Hague are being collected for MLST typing. These results will show whether new highly prevalent clones are emerging and whether the known prevalent clones are still spread-

ing. Furthermore, although there was no difference in clinical outcome for patients infected with ST406 there was an increased use of inhaled antibiotics, which indicates that continued (prospective) monitoring of the effects of highly prevalent clones is warranted. Possibly a more prolonged observation period is necessary to identify differences in clinical outcome between ST406 infected patients and others in a large CF population. If future research continues to show no detrimental effect of such clones, it might lead to a revision of the individual segregation policy, e.g. by investigating the possibility of cohort segregation, which is advocated in the UK[6]. Persistence of the highly prevalent clone ST406 is high, which would imply that upon cohort segregation of these patients, not much is likely to change in *P. aeruginosa* genotypes in these patients. Under ongoing molecular epidemiologic monitoring of *P. aeruginosa* genotype, the feasibility of contact between patients within cohorts (infected with a highly prevalent clone, infected with a sporadic clone, not *P. aeruginosa* infected) might be investigated. In these studies, the effect of other pathogens on the dominance and persistence of the clone could also be taken into account, as these might offer new insight into the reason for its increased persistence.

Finally, although cohort segregation measures have generally been met with support and understanding by patients and their parents[7;8], data are lacking on the psychosocial burden of individual segregation. The fact that a group of teenage CF patients are organising a yearly one-day event for CF patients described in Chapter 5 indicates that there is still a need for contact with other CF patients among many patients. Moreover, it would be interesting to study whether the psychosocial burden of the segregation policy still applies to parents and their children who have been diagnosed through the recently implemented newborn screening for CF. It is conceivable that these patients have a different view of contact with other CF patients, as they have not experienced the situation before the segregation policy was implemented and they might be more accepting of this policy. Data are lacking on the psychosocial burden for this new group of patients.

CONCLUSIONS

In conclusion, pathogens continue to play an important role in CF lung disease and many new and emerging pathogens are reported. The individual effects of certain emerging pathogens on the course of lung disease are minimal when performing longitudinal follow-up and taking into account possible confounding factors. For highly prevalent clones however, continuing prospective monitoring of clinical impact is necessary, to study the long term effects and to evaluate the current infection control policy. Furthermore, the emergence of antibiotic resistance in relation to antibiotic treatment warrants caution and the search for new and improved antibiotic treatment strategies. In general, while previous focus in CF research has been on single pathogen infections, the current perspective is shifting towards polymicrobial infections. Thus future research should focus on the role of polymicrobial infections on lung disease, pathogen virulence and antibiotic response.

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Chapter 11

DUTCH SUMMARY IN PLAIN LANGUAGE

Nederlandse samenvatting in begrijpelijke taal

WAT IS CYSTIC FIBROSIS?

Cystic fibrosis (CF), ook wel taaislijmziekte genoemd, is de meest voorkomende erfelijke aandoening onder de Westerse bevolking. CF wordt veroorzaakt door een mutatie in een gen dat voor het CFTR-eiwit codeert. Dit eiwit doet dienst als chloorkanaal in cellen van het epitheel (de bovenste laag van bijvoorbeeld huid en slijmvliezen). De mutatie leidt tot minder of slecht functionerend eiwit, waardoor er een verstoring optreedt in het transport van chloor, natrIUm en water in de slijmvliezen. Als gevolg hiervan is er abnormaal taai slijm in de luchtwegen, de alvleesklier, lever en darmen. Hierdoor ontstaan er slijmophopingen in het verteringsstelsel die o.a. suikerziekte en darmobstructies tot gevolg kunnen hebben. Door het taaie slijm in de luchtwegen kunnen bacteriën minder goed worden afgevoerd en ook ontstaan er slijmophopingen in de longen, wat leidt tot chronische infectie. Uiteindelijk resulteert dit in longschade en terminaal longfalen; dit is de belangrijkste doodsoorzaak bij CF.

CF is een zogenaamde autosomaal recessieve aandoening. Dit betekent dat iemand de ziekte heeft als hij van beide ouders een gen met een mutatie doorgegeven krijgt. In Nederland is ongeveer 1 op de 30 mensen drager van één CF-mutatie (zonder dus zelf de ziekte te hebben) en heeft 1 op de 4750 levend geboren baby's de ziekte. Momenteel zijn er zo'n 1300 CF patiënten in Nederland. De overleving van CF patiënten is de afgelopen decennia drastisch gestegen. Waar vroeger de meerderheid van de kinderen de volwassenheid niet haalde, is de gemiddelde levensverwachting nu 35 to 40 jaar.

LUCHTWEGPATHOGENEN BIJ CF

De belangrijkste problemen bij CF spelen zich af in de longen. Waarschijnlijk voornamelijk door het taaie slijm, maar ook door andere mogelijke oorzaken zoals een verhoogde vatbaarheid voor bepaalde bacteriën, speelt chronische infectie een belangrijke rol. De meest voorkomende bacterie hierbij is *Pseudomonas aeruginosa*, waarmee zo'n 60-80% van de volwassen CF patiënten chronisch geïnfecteerd raakt. *P. aeruginosa* is een bacterie die overal om ons heen aanwezig is en zich vooral bevindt in waterige omgevingen zoals bijvoorbeeld in het doucheputje of in een bloemenvaas. Bij gezonde mensen veroorzaakt *P. aeruginosa* zelden een infectie. Waarom zoveel CF patiënten met deze bacterie geïnfecteerd raken is nog niet helemaal duidelijk. Daarnaast zijn er veel andere micro-organismen die een rol lijken te spelen bij CF. Het is echter niet altijd duidelijk of micro-

organismen die worden gevonden bij CF patiënten ook daadwerkelijk een aandeel hebben in het ziekteproces. In hoofdstuk 2 beschrijven we een aantal van deze micro-organismen en in hoeverre deze invloed hebben op de klinische toestand.

VIRUSSEN

Naast bacteriën lijkt het erop dat ook virussen belangrijk kunnen zijn bij CF. Uit verschillende onderzoeken wordt gesuggereerd dat luchtwegvirussen een faciliterende rol kunnen spelen bij het ontstaan van bacteriële infecties. Meer specifiek is uit laboratorIUmonderzoek gebleken dat een veel voorkomend luchtwegvirus (RSV) de *P. aeruginosa* bacterie kan helpen met hechten aan luchtwegcellen. In hoodstuk 3 laten we zien dat in een muismodel waarbij er sprake is van een gelijktijdige RSV infectie, de muizen een ernstiger *P. aeruginosa* luchtweginfectie hebben dan wanneer de muizen alleen met *P. aeruginosa* worden geïnfecteerd. De aanwezigheid van luchtwegvirussen draagt dus mogelijk bij aan het geïnfecteerd raken met *P. aeruginosa.* Daarom is het belangrijk om ook bij CF patiënten aandacht te hebben voor virusinfecties omdat tijdens die infecties bacteriën zich makkelijker zouden kunnen nestelen. Toekomstig onderzoek moet uitwijzen op wat voor manier patiënten het beste behandeld kunnen worden tijdens virusinfecties om dit te voorkomen.

SCHIMMELS

Schimmels worden in toenemende mate aangetoond in de luchtwegen van CF patiënten. Een voorbeeld van een veel voorkomende schimmel is *Aspergillus fumigatus*, die bij astma-en CF-patiënten tot een allergisch beeld kan lijden (ABPA, allergische bronchopulmonale aspergillose). Echter de rol van deze schimmel in de afwezigheid van ABPA is onduidelijk. In hoofdstuk 4 beschrijven we een onderzoek waarbij we patiënten met de schimmel in hun luchtwegkweken (maar zónder ABPA) vergelijken met patiënten zonder de schimmel. Over een periode van vijf jaar is er geen verschil te vinden in het beloop van de longfunctie tussen deze groepen patiënten. Dit suggereert dat de aanwezigheid van de schimmel op zich waarschijnlijk niet veel schade aanricht. Deze resultaten suggereren dat het effect van deze schimmel op groepsniveau niet groot is, maar het zou kunnen dan in individuele gevallen er wel een effect is van *A. fumigatus*.

'HOOG PREVALENTE' P. AERUGINOSA STAMMEN

De bacterie *P. aeruginosa* blijft het meest voorkomend bij CF patiënten. Men dacht altijd dat elke CF patiënt een uniek *P. aeruginosa* genotype bij zich zou dragen, m.a.w. geïnfecteerd raakt met een unieke 'stam' die opgedaan wordt uit de omgeving. Hierbij was het idee dat het delen van stammen alleen voorkwam bij huisgenoten, die intensief contact met elkaar hebben. Echter, in de jaren negentig verschenen verschillende rapporten van zogenaamde 'veel voorkomende' of hoog prevalente stammen: dit zijn stammen die door meerdere, onverwante mensen gedeeld worden. Het vóórkomen van deze stammen in verschillende CF centra in bijvoorbeeld het Verenigd Koninkrijk en Australië suggereerde dat deze stammen werden overgedragen tussen patiënten. Omdat in veel van deze rapporten een associatie werd gevonden tussen een slechtere klinische uitkomst en het hebben van zo'n stam, besloot men om het infectiepreventie beleid aan te passen. In Nederland heeft men daarom in 2005 een segregatiebeleid ingevoerd. Dit houdt in dat CF patiënten zowel in het ziekenhuis als op de polikliniek gescheiden worden behandeld. Buiten het ziekenhuis wordt hen afgeraden fysiek contact te hebben met andere CF-patiënten. Daarnaast worden patiënt-ontmoetingsevenementen zoals zomerkampen niet meer georganiseerd. Pas in 2007 werd een groot onderzoek gedaan naar het vóórkomen van een dergelijke Nederlandse stam. Inderdaad werd in de populatie van het CF centrum in Utrecht en Den Haag een hoog prevalente stam gevonden, die ST406 werd genoemd. In dit proefschrift onderzoeken wij of deze recent ontdekte, Nederlandse stam, net als de andere veel voorkomende stammen ook geassocieerd is met een slechtere uitkomst. In hoofdstuk 6, 7 en 8 laten we zien dat het geïnfecteerd zijn met de ST406 stam niet geassocieerd is met een slechtere longfunctie, een slechter longfunctiebeloop of een grotere kans op sterfte of longtransplantatie. Het lijkt erop dat infectie met deze stam dus, anders dan bepaalde andere hoog prevalente stammen, niet gepaard gaat met een ernstiger beloop van de ziekte. Wat wel opviel was dat mensen met ST406 vaker werden behandeld met onderhouds inhalatieantibiotica. Dit zou erop kunnen wijzen dat er toch meer behandeling nodig is bij deze patiënten, maar dat dit verschil nog niet heeft geleid tot een duidelijk verschil in ziekte. In hoofdstuk 7 beschrijven we bovendien dat de ST406 stam zich over een periode van tien jaar significant beter handhaaft in patiënten dan andere stammen; andere stammen worden vaker vervangen door een nieuwe stam. Het lijkt dus wel een stam te zijn die moeilijker weg te krijgen is. Het is belangrijk om de klinische gevolgen van deze stam daarom goed te blijven onderzoeken.

Als inderdaad blijkt dat ook in de toekomst patiënten met dit soort stammen er niet slechter aan toe zijn dan andere patiënten, dan zou een eventuele verandering van het segregatiebeleid onderzocht kunnen worden.

Ondanks het advies om elkaar niet fysiek te ontmoeten, is er een groep jonge CF-patiënten die sinds 2005 jaarlijks een eendaags strandevenement organiseert voor CF-patiënten: het CF Beach Dance evenement. Men koos voor een openlucht evenement vanuit de gedachte dat bij een korte ontmoeting in de openlucht de kans op overdracht van *P. aeruginosa* tussen patiënten klein is. In hoofdstuk 5 beschrijven we een onderzoek waarbij we tijdens CFBD onderzoeken óf en in hoeverre er sprake is van de overdracht van *P. aeruginosa*. Er werden tijdens het evenement twee mogelijke gevallen van overdracht gevonden. Echter door het zeer lage aantal bezoekers dat jaar en door de oververtegenwoordiging van de hoog prevalente ST406 stam is het niet goed mogelijk hier conclusies uit te trekken.

ANTIBIOTICARESISTENTIE

De verbetering van behandeling van luchtweginfecties bij CF patiënten in de afgelopen jaren heeft geleid tot een drastische toename van de levensverwachting. Het vroegtijdig behandelen van patiënten die geïnfecteerd zijn met *P. aeruginosa* heeft hier waarschijnlijk een belangrijk aandeel in gehad. Echter er zijn ook aanwijzingen dat er een opkomst is van (multi) resistente bacteriën. Dit zijn bacteriën die ongevoelig zijn geworden voor antibiotica. In hoofdstuk 9 beschrijven we een onderzoek waarin we kijken naar de relatie tussen het langdurig gebruik van inhalatie antibiotica en de aanwezigheid van resistente *P. aeruginosa* stammen. Het risico op het krijgen van een resistente *P. aeruginosa* stam blijkt significant hoger bij patiënten wanneer zij inhalatie tobramycine gebruiken. Het is overigens niet zo dat deze patiënten vervolgens direct een sterkere daling laten zien in hun longfunctie. Deze gegevens laten wel zien dat resistente stammen optreden bij langdurig tobramycine gebruik en dat het belangrijk is om hier onderzoek naar te blijven doen. Het valt aan te nemen dat in de toekomst, met de toenemende stijging van de levensverwachting, patiënten langer zullen worden behandeld met onderhoudsantibiotica en dat de kans op resistentie groter zou kunnen worden.

CONCLUSIE

Samenvattend laten we zien dat micro-organismen in de luchtwegen een belangrijke rol spelen bij CF patiënten. Van sommige opkomende micro-organismen is het niet geheel duidelijk of ze bijdragen aan een verergering van de ziekte. Uit longitudinaal onderzoek laten wij zien dat *A. fumigatus* niet leidt tot een snellere longfunctiedaling over een periode van vijf jaar. Verder laten we zien dat er interactie kan plaatsvinden tussen pathogenen, zoals RSV en *P. aeruginosa*. Tenslotte onderzochten we het effect van een recent ontdekte, hoog-prevalente *P. aeruginosa* stam. Anders dan rapporten uit het buitenland gaat infectie met deze stam niet gepaard met een slechtere klinische toestand of meer kans of sterfte of longtransplantatie. Echter de stam was wel in staat langer ter persisteren en patiënten gebruikten vaker onderhoudsantibiotica. Daarom is het belangrijk de effecten van deze stam te blijven onderzoeken. Wellicht wordt in de toekomst het lange-termijn effect van deze stam duidelijk en kan dan worden beoordeeld of het segregatiebeleid mogelijk kan worden veranderd.Ten slotte toonden wij dat langdurig gebruik van een inhalatie antibioticum als tobramycine, de kans op resistentie kan doen toenemen. Het is dus belangrijk om meer verschillende antibiotica te ontwikkelen en toe te passen die geschikt zijn voor inhalatie, om zo resistentie bij langdurig gebruik van één middel te voorkomen.

Dankwoord

Hoewel er maar één naam op dit boekje staat hebben veel meer mensen op enige wijze bijgedragen aan de totstandkoming van dit proefschrift. Mijn dank aan hen is groot. Een aantal mensen wil ik in het bijzonder noemen.

Allereerst wil ik de CF-patiënten en hun ouders bedanken die deel hebben genomen aan de verschillende onderzoeken. Ik heb veel bewondering voor jullie bereidheid om mee te werken, wetende dat de impact van CF op een mensenleven groot is. Daarnaast ben ik de Nederlandse Cystic Fibrosis Stichting (NCFS) zeer erkentelijk voor de financiële steun die zij aan de verschillende projecten hebben verleend.

De leden van de leescommissie, Prof. dr. J.W.J. Lammers, Prof. dr. A.M. van Furth, Prof. dr. E.A.M. Sanders, Prof. dr. H.A.W.M. Tiddens en Prof. dr. A.J. van Vught wil ik bedanken voor het beoordelen van het manuscript.

Beste Kors, bedankt voor de vele mogelijkheden die je me hebt geboden gedurende de afgelopen jaren. Al tijdens mijn onderzoeksstage werd ik aangestoken door je enthousiasme voor CF-onderzoek en ik ben erg dankbaar voor de mogelijkheid om na mijn studie als arts-onderzoeker te komen werken. Ik vond het geweldig dat ik een jaar in Kopenhagen aan de slag kon, en heb daar en in de jaren daarna meer geleerd dan waar ik ooit op had kunnen hopen. Bedankt ook voor je steun bij het toewerken naar de opleiding.

Beste Marc, na het eerste jaar van mijn onderzoek begon de samenwerking met de afdeling microbiologie. Ik waardeer de tijd die je vrij maakte om mee te denken over analyses of kritisch naar mijn stukken te kijken. Bedankt dat je me de gelegenheid gaf om zoveel te leren van de epidemiologie van infectieziekten.

Beste Tom, bedankt voor je zeer betrokken begeleiding de afgelopen jaren als co-promotor. Niet alleen was je, ondanks drukte in de kliniek, altijd bereid het hoofd te buigen over vragen die ik had (pen en papier in de aanslag, druk meerekenend) maar ook kwam je regelmatig de flexkamer binnen lopen om even te kletsen. Ik waardeer je enorme attentheid en je steun zowel in het onderzoek zelf als bij de aanloop naar de opleiding.

Dear Helle, thank you very much for your guidance and supervision during my time in Copenhagen. Despite your busy schedule you always had time for discussions about our experiments and for answering my questions. Your hospitality is exceptional, and I greatly enjoyed the many dinners with you and your family. Thank you for making my time in Copenhagen successful and memorable.

Dear Oana, thank you for your extensive assistance with experiments at the animal department. You were always available for help with interpreting results and designing new experiments. Also, thanks for your hospitality and for lending us your husband's bike, which proved to be an essential asset in Copenhagen.

Dear Niels, thank you very much for giving me the opportunity to work in your lab. I enjoyed working in an environment where knowledge of *P. aeruginosa* is so abundant. Also I very much appreciate the opportunities you've given me to further develop my scientific career, like taking the laboratory animal science course and going to the ASM conference in Boston.

Dear Steen, Niels-Erik, Hanne-Louise, thank you for your help in performing the pathology studies of our experiments. I greatly appreciate the brilliant histopathology images you created and the many BAL'S you performed on our mice.

Dear Tina, thank you for your technical assistance in our experiments. All others at the lab at Panum: Lotte, Baoleri, Hanne, Jette, thanks for making me feel welcome and for all the tea and Friday bread sessions. Ulla and Nikolai from the microbiology department at Rigshospitalet, thanks for your technical assistance. Søren, thank you for giving me the opportunity to learn about evolution of *P. aeruginosa* at DTU and for many inspiring discussions. Mustafa, thanks for being our friend and for the many great chats over coffee, beer or cocktails during our time in Copenhagen.

Collega's van de kinderlongziekten: bedankt voor de fijne tijd als lid van de kinderlongziekten-familie. De ontelbare taartmomenten, maar ook het kerstontbijt en teamuitjes waren stuk voor stuk weer kleine hoogtepunten in het leven van een onderzoeker (en vroegen om een hoop balansdagen). Myriam en Sylvia, heel veel dank voor jullie hulp bij allerlei logistieke zaken en meer. Ik ben onder de indruk van hoe jullie ondanks de drukke bezigheden altijd tijd vrij kunnen maken voor een praatje. Rolien, dankjewel voor je onmisbare hulp bij CFBD. Arts-onderzoekers: Gerdien, Marije, Pauline, Anne, Kim, Jacobien en Nicole: bedankt voor de gezelligheid, zowel op het werk als daarbuiten. Heel veel succes met jullie onderzoek. Pauline, dank voor de prettige samenwerking en de lol tijdens onze weekjes in Kopenhagen. Marije, achterbuuf, bedankt voor je adviezen bij ingewikkelde statistiek en menig andere perikelen. Gerdien, dankjewel voor de inspirerende besprekingen die we her en der hadden, voor de gezellige (shisha-)avonden in Kopenhagen en voor de spannende zeiltocht vorig jaar. Geweldig dat onze rollen nu omgedraaid zijn en je bij mijn verdediging naast me staat als paranimf.

Annick, Judith, Sanne N, Sanne H, Eva, Gerwin, Arjan, Evelien, Coralie, Sabine en menig andere bewoner van de flexkamer. Binnen de kamer heeft zich een heuse cultuur gevormd, met eigen rituelen (You Tube of the day), tradities (lelijke magneten meenemen na een reis) en zelfs een eigen (straat)taal. Bedankt dat ik hier drie jaar met veel plezier kon 'wonen' en mijzelf kon zijn. Samen met de bewoners van de buurkamer (Korneel, Stefan, Joost en Thijs) maakten jullie het onderzoekersleven tot een klein feest.

Beste Hubert, dankjewel voor het helpen met de data en het meedenken met het resistentie-stuk.

Beste Veronica, bedankt voor je hulp bij het opzetten van de CFBD studie. Ik heb veel bewondering voor je inzet bij dit soort activiteiten.

Beste Rebecca, bedankt voor je uitgebreide hulp bij verschillende statistische analyses. Bij mijn vele vragen formuleerde jij je antwoorden altijd zo helder, dat zelfs ingewikkelde analyses voor mij begrijpelijk werden. Dank voor de tijd die je elke keer vrijmaakte.

Van de afdeling longziekten wil ik Ferdinand Teding van Berkhout en Pieter Zanen bedanken voor het aanleveren van de longfunctie-data van de volwassenen.

Beste Rosa, bedankt voor de prettige samenwerking in de afgelopen jaren. Je hebt me veel geleerd over typeren en ik heb genoten van de paar weken samen pipetteren op de technische universiteit in Kopenhagen. Het toppunt van onze samenwerking was toch wel tijdens CF Beach Dance, toen we midden in de nacht in de longfunctiebus terug moesten vanaf Bloemendaal, met groot licht aan en ternauwernood strandvolk ontwijkend, om vervolgens nog hoestwatsamples in te zetten op het lab. Ook de vele besprekingen die we hadden vond ik erg inspirerend. Veel succes met de afronding van je onderzoek.

Beste Piet, bedankt voor je hulp bij het opzetten van het co-infectiemodel. Het was geweldig dat je ons de eerste dagen in Kopenhagen kwam helpen en daarna ook altijd bereid was mee te denken. Bedankt ook voor het opsturen van RSV en XL-hagelslag.

Beste Rob, veel dank voor de samenwerking en je bereidheid om me dingen uit te leggen over typeren. Je deed dat altijd zó duidelijk en met zoveel precisie, dat ik het zowaar begon te snappen.

Medewerkers op het lab: dank voor jullie hulp bij de TROPIC- en de FOK studie. Lenie en Hetty, erg bedankt voor het extraheren van de kweekdata met behulp van complexe queries en scripts. Mede-onderzoekers bij de XEWMM: ik vond het geweldig om van jullie te leren tijdens de 'extremely early Wednesday morning meetings'. Bedankt voor het meedenken met de analyses en veel succes met jullie onderzoek.

Haga Ziekenhuis Den Haag: Harry, veel dank voor je hulp en de samenwerking bij de verschillende gezamenlijke projecten. Annemarie en Jane, bedankt voor jullie hulp bij het verzamelen van de data. Roland, bedankt voor de samenwerking in de FOK-studie.

De volgende mensen wil ik bedanken voor hun hulp bij het verzamelen van data voor de FOK-studie: Dorien Holtslag (AZM), Els van der Wiel (Erasmus MC), Ron Meijer (Erasmus MC), Irma Bon (VUMC), Maaike van Brederode (AMC) en Bart Rottier (UMCG).

Collega arts-assistenten, kinderartsen en andere medewerkers van de afdeling kindergeneeskunde van het St. Antonius ziekenhuis, Nieuwegein: bedankt voor de prettige werksfeer en dat jullie me de kans geven om in korte tijd zóveel te leren.

Lieve Irene T, Judith, Lara, Mariëlle, Irene D, Lisette, Eline, Rianne, Christine, Evelien, Dennis, Sjoerd, Alexis & Joost, Bart & Ingrid, Maarten & Anne, Hessel & Saskia en anderen die ik vergeet: dank voor het broodnodige plezier buiten het werk en jullie interesse en steun tijdens mijn onderzoekstijd. Mariëlle, bedankt dat je mijn paranimf wilt zijn en naast me staat bij de verdediging.

Lieve familie, Peter & Ria, dank voor jullie belangstelling en gezelligheid tijdens familie-bijeenkomsten. Untuk keluarga yang di Surabaya, saya ingin mengucapkan terima kasih atas perhatian dan dukungan moril. Dear Novi and Paco, thanks for coming over to be at my defense, and thanks Novi for being like an overseas sister.

Lieve Ben, Nicolette, Maggie en Ties: het is heerlijk om je zo thuis te voelen bij je schoonfamilie. Veel dank voor jullie warmte en gezelligheid. Ties, ik ben er erg trots op dat BUROPONY de vormgeving van dit proefschrift heeft gedaan, veel dank!

Lieve pa en ma, bij elke keuze die ik maakte hebben jullie achter mij gestaan. Dit waardeer ik enorm en dit maakt een heleboel mogelijk. Dank voor jullie nooit aflatende steun en interesse. Lieve Jacintha, dank voor de lol die we samen hebben en je luisterend oor. Wij begrijpen elkaar.

Lieve Kars, door de gedrevenheid en creativiteit die jij in je werk laat zien heb ik me vaak laten inspireren. Ik vind het nog steeds geweldig dat je zomaar met me meeging naar Kopenhagen. Mede daardoor was dat een fantastische tijd. Maar bovenal: dankjewel voor wie je bent!

Lieke de Vrankrijker,
Utrecht, oktober 2011.

CURRICULUM VITAE

Lieke de Vrankrijker werd op 28 april 1981 geboren te Woerden. Ze groeide op in Montfoort en haalde in 1999 haar atheneum diploma aan het Minkema College te Woerden. Aansluitend begon zij aan de studie psychologie aan de Universiteit Utrecht. In 2000 werd zij ingeloot voor geneeskunde aan dezelfde universiteit. In 2004 liep zij haar co-schap kindergeneeskunde in het Dr. Soetomo ziekenhuis van de Airlangga Universiteit in Surabaya, Indonesië. Tijdens het laatste jaar van haar opleiding deed zij wetenschappelijk onderzoek naar cystic fibrosis bij de afdeling kinderlongziekten onder leiding van Prof. dr. C.K. van der Ent en liep zij haar semi-arts stage op de afdeling hematologie/immunologie, beide in het Wilhelmina Kinderziekenhuis te Utrecht. Na haar afstuderen in 2006 werkte zij vijf maanden als ANIOS in het WKZ. In mei 2007 begon zij aan het promotie-onderzoek dat uiteindelijk tot dit proefschrift heeft geleid (onder begeleiding van Prof. dr. C.K. van der Ent en Prof. dr. T.F.W. Wolfs). Tussen juni 2007 en juli 2008 deed zij in dit kader laboratorium-onderzoek bij de afdeling medische microbiologie van het Rigshospitalet in Kopenhagen, onder begeleiding van Prof. dr. N. Høiby en Dr. H.K. Johansen. Na terugkomst verrichtte zij epidemiologisch onderzoek, in nauwe samenwerking met de afdeling Medische Microbiologie (onder begeleiding van Prof. dr. M.J.M. Bonten). In oktober 2011 startte zij aan de klinische opleiding tot kinderarts vanuit het Wilhelmina Kinderziekenhuis (opleider Dr. J. Frenkel). Momenteel werkt zij als arts-assistent kindergeneeskunde in het St. Antonius Ziekenhuis te Nieuwegein voor het perifere deel van haar opleiding (opleider Dr. W.A.F. Balemans). Lieke woont samen met Kars Alfrink.

LIST OF PUBLICATIONS

THIS THESIS

A.M.M. de Vrankrijker, R. van Mansfeld, R.W. Brimicombe, T.F.W. Wolfs, H.G.M. Heijerman, F. Teding van Berkhout, R.J.L. Willems, E.J.M. Weersink, G. Wesseling, R.D.B. Meijer, B.L. Rottier, M.J.M. Bonten, C.K. van der Ent. Increased persistence of a highly prevalent *Pseudomonas aeruginosa* clone found in cystic fibrosis patients. *Submitted.*

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