Pseudomonas aeruginosa infections in children with cystic fibrosis

determinants, detection and directed intervention

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Pseudomonas aeruginosa infecties bij kinderen met cystic fibrosis
bacteriële karakteristieken, opsporing en interventie
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 11 februari 2010 des middags te 4.15 uur

door

Geertje Adriana Tramper-Stranders
geboren op 17 mei 1977 te Yerseke (Reimerswaal)

Promotoren: Prof.dr. J.L.L. Kimpen
Prof.dr. C.K. van der Ent

Co-promotor: Dr. T.F.W. Wolfs

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What if...

Nineteen hundred seventy seven, the year I was born. What if I was born with cystic fibrosis? I would have had a mere fifty percent chance of survival beyond an age of thirty years old.[1] Possibly I could have written this thesis, but more likely I couldn't. The mean life expectancy of a newborn with cystic fibrosis has nowadays increased to around fourty years old. During my own lifetime, some great insights into the pathogenesis of cystic fibrosis were achieved and successful interventions were established leading to an improved survival. Nevertheless, the more insight we have into the complexity of the disease, the more gaps we discover to be filled in. The human body remains a miracle, but the spectrum of therapeutic options has greatly improved over the last decades.

What if micro-organisms weren't that smart in escaping our immunity system or therapy, thus making the treatment much more easy? Although many infectious diseases can now be prevented - by either proper hygiene and vaccinations - or treated, micro-organisms still pose us for a continuous challenge by alteration of their properties. It's like a love-hate relationship: we often fight them, yet we can't live without (some of) them. The question '*who am I*' could perhaps better be replaced by *'who are we*'?[2]

What if patients and their families would not see the need to cooperate in research in order to achieve greater insights? Instead their willingness was enormous, although their personal short-term benefit remained unclear. We are very grateful for their participation. Interestingly, researching new therapeutic treatments is not something of the last 'evidence based medicine' decades. The forerunner of the clinical trial was already mentioned about one thousand years ago by the famous Persian physician Ibn-Sina (Avicenna). His impressive Canon of Medicine thoroughly describes testing drugs while considering the ethical and methodological conditions. This work became a standard in medieval medical schools in Europe and in the East. Although the nature of many diseases was still unknown, medical treatments were developed for a considerable amount of diseases.[3]

critical reflection | fun | inspiring environment | creativity | stimulating talks | confidence | refreshing thoughts | serious business | rest | assistance

What if the foregoing conditions were not offered by my supervisors, colleagues, friends, family and partner...? Many things in my life, including performing this study, were enabled by people surrounding me, for which I am very grateful.

I especially want to thank Tom Wolfs, Kors van der Ent and Jan Kimpen for supervising me during this project. Our inspirational meetings always encouraged me to get the best out of myself. Lastly, thank you Jonathan for who you are and especially for our successful collaboration in creating this book!

Gerdien

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2. Editoral. *Nature* 2008;453:563

3. Sajadi, MM et al. Ibn Sina and the clinical trial. *Annals of Internal Medicine* 2009;150;640-3

I was born to be with You
In this space and time
After that and ever after I haven't had a clue
Only to break rhyme
This foolishness can leave a heart black and blue

Only love can leave such a mark
But only love can heal such a scar

I was born to sing for You
I didn't have a choice but to lift You up
And sing whatever song You wanted me to
I give You back my voice
From the womb my first cry
It was a joyful noise

Only love, only love can leave such a mark
But only love, only love can heal such a scar

Justified till we die
You and I will magnify
The Magnificent
Magnificent

Magnificent (U2), based on the Magnificat

In memory of Elsbeth Schaap (1977-2004), dear friend, oncology nurse and promising researcher

Girl - Rajasthan, India

1

Introduction

General information about cystic fibrosis

General information about cystic fibrosis

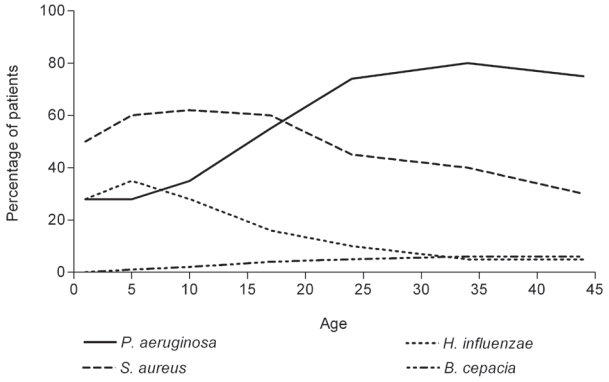
Cystic fibrosis

Cystic fibrosis (CF) is an inherited autosomal recessive disease common in the Caucasian population. CF is the result of mutations in the cystic fibrosis transmembrane regulator (CFTR) gene on chromosome 7. Mutant CFTR leads to absent, deficient or misfolded CFTR protein resulting in disturbed regulation of ion- and fluid homeostasis on epithelial surfaces.[1;2]
Cystic fibrosis primarily affects the respiratory, gastro-intestinal and reproductive system. The overall birth prevalence in the Netherlands is about 1 in 4750 newborns.[3] Mean life expectancy, which is nowadays about 37 years, has drastically increased last decades but is mainly determined by respiratory failure because of chronic respiratory infections and accompanying, primarily neutrophil dominated, inflammatory response.[4]

Respiratory infections in patients with cystic fibrosis

Both viral, bacterial and fungal pathogens are responsible for respiratory infections among children with CF. However, chronic infection with bacterial pathogens is thought to be the major determinant of lung function in children with CF. Viral respiratory tract infection often precedes bacterial infection; there seems to be an interaction between viruses and bacteria promoting bacterial colonisation.[5] Bacterial pathogens involved in respiratory infections in infants with CF are mainly *Staphylococcus aureus* and non-typable *Haemophilus influenzae*.[4] Subsequently, many children become infected with *Pseudomonas aeruginosa*. As determined by culture of respiratory secretions, 28-85% of patients ever demonstrated *P. aeruginosa* during their first 3 years of life, depending on the isolation method.[6-8] The percentage of recurrent or chronic *P. aeruginosa* infection increases with age (figure 1). Chronic infections with *P. aeruginosa* accelerate the progression to irreversible pulmonary damage and are a major predictor of morbidity and mortality.[9-11]

Figure 1 Prevalence of respiratory infections per age, according to CF patient registry, USA 2006



Various host conditions predispose for the acquisition of *P. aeruginosa* infection among which CFTR-genotype, CF associated gastro-intestinal abnormalities, hospitalisation and exposure to other CF patients, isolation of *S. aureus* and long-term prophylaxis with antistaphylococcal agents.[12-14] Several hypotheses have been formulated to understand the susceptibility of the CF lung for chronic infections, especially with *P. aeruginosa*. First, a reduced production of airway surface liquid and decreased transport of dehydrated mucus leads to inhibited bacterial clearance.[2] Secondly, local host antimicrobial peptides have a diminished function due to the high airway salt concentration. Furthermore, increased adherence of *P. aeruginosa* to epithelial cells is suggested because of overexpression of asialoganglioside receptors in cells with mutant CTFR. Additionally, some authors claim that the normal CFTR protein functions as a receptor that is associated with internalisation of *P. aeruginosa*, where after *P. aeruginosa* is cleared by innate immunity effector proteins. Mutant CFTR might not bind pathogens, leaving bacteria free to multiply in the airways.[2;15;16]

The bacteria *'Pseudomonas aeruginosa'* and cystic fibrosis

Pseudomonad literally means 'false unit', being derived from the Greek pseudo (ψευδο 'false') and monas (μονάς / μονάδα 'a single unit'). The term "monad" was used in the early history of microbiology to denote single-celled organisms. Because of their widespread occurrence in nature, the pseudomonads were observed early in the history of microbiology. The generic name Pseudomonas created for these organisms was defined in rather vague terms in 1894 as a genus of Gram-negative, rod-shaped and polar-flagella bacteria.[17]

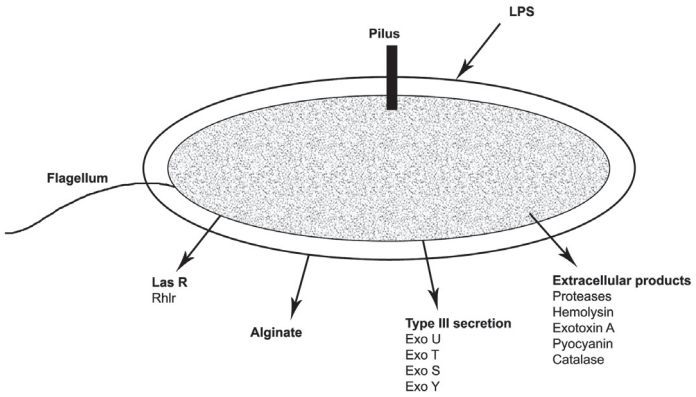
A smart settler

The large genome of *P. aeruginosa* with its genetic flexibility is its key to success.[16] Once *P. aeruginosa* infects the lungs, it exhibits several ways of escaping host and antimicrobial defences by expression and secretion of virulence factors. An important bacterial system for regulation of virulence factor expression and cell- to cell signalling is the quorum sensing system. This system is regulated by auto-inducer molecules called acyl homoserine lactones.[16;18;19] Virulence factors that are under control of the quorum sensing system are both cell-associated (flagella, pili, alginate/biofilm, lipopolysaccharide) and extracellular virulence factors (proteases, hemolysins, exotoxin A, exoenzyme S, pyocyanin) (figure 2).

In patients with CF, *P. aeruginosa* often grows in a biofilm mode of growth established by excretion of alginate, an extracellular polysaccharide. Penetration of antibiotics and influx of neutrophils into the biofilm is hampered, preventing phagocytic clearance of bacteria.[20] The presence of an anaerobic condition in the viscous mucus has been suggested to increase alginate production. Initial colonisation and adherence to epithelial cells is regulated by pili and flagella, which are highly antigenic.[16] Flagella are primarily responsible for bacterial motility but do also have the capacity to adhere to epithelial cells. Cilial transport, which has a major function in clearance of mucus, is inhibited by pyocyanin. Cytotoxicity and

subsequent tissue destruction is facilitated by the type III secretion system products such as exoenzyme S or U. The type III secretion system is responsible for bacterial invasion in eukaryotic cells by injecting toxins directly into these cells.

Figure 2 Virulence factors of *P. aeruginosa*[16]



Tissue invasion is also promoted by exotoxin A, which facilitates the dissemination of infection. Specific components of human pulmonary tissue, e.g. the protein elastin, are a major target for the proteases.[16] Additionally, there is some evidence that the quorum sensing system induces apoptosis directly in neutrophils and macrophages.[21] Extracellular products such as the proteases are able to degrade immunoglobulin and complement protein. This is one of the reasons that host anti-*Pseudomonas* antibodies fail to mediate killing of *P. aeruginosa.*

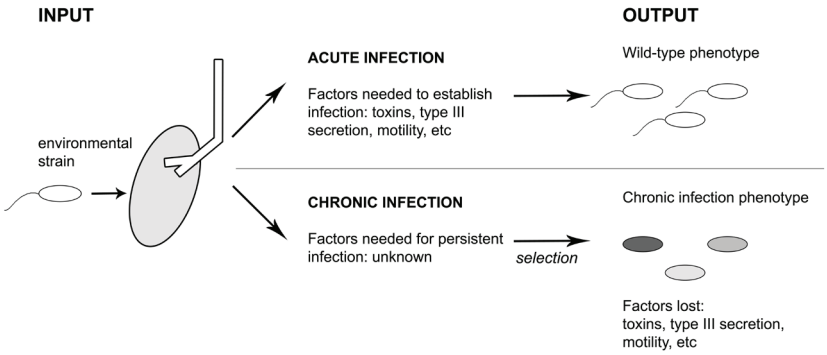
In conclusion, a combination of bacterial effects directed at human lung tissue and at host immune response facilitates infection with *P. aeruginosa* in the light of a susceptible environment such as the CF lung.

Epidemiology

P. aeruginosa isolates detected at the moment of initial infection are often highly virulent, environmental isolates. With time, these isolates adapt genetically to the CF lung with decreased expression of virulence factors, promoting survival of bacteria (figure 3).[22-24]

Patients with CF with chronic *P. aeruginosa* infection often share common bacterial genotypes that are either selected or transmitted among patients, so-called 'epidemic strains' or 'clones'. Several distinct clonal genotypes have been demonstrated among patients with CF in different countries. The clinical significance of clonal strains remains uncertain.[25-27] There is no clear relation between increased or diminished virulence or bacterial resistance and clonality; these clonal genotypes might probably benefit from so far not yet elucidated pathways.[28]

Figure 3 *P. aeruginosa* in initial and chronic infection[22]



Therapeutic strategies to attack *P. aeruginosa* in patients with cystic fibrosis

Antimicrobial therapy

P. aeruginosa is inherently resistant to many antibiotics. Antibiotic penetration into bronchial mucosa and mucus is diminished because of airway scarring. Consequently, several routes of delivering antibiotics are practised, *ie.* oral, intravenous and inhalation therapy. Current therapy strategies are aimed at prevention of chronic *P. aeruginosa* infection and subsequent pulmonary function decline.[4;19] Initial *P. aeruginosa* infection is nowadays managed with intensive antimicrobial therapy, often a combination of oral or intravenous antibiotics with inhaled antibiotics for a prolonged period. A delay in the onset of chronic *P. aeruginosa* infection or eradication of the pathogen from the CF airways might be achieved when antibiotic treatment is initiated immediately after pulmonary colonisation.[29-32] However, interpretation of study results has been hampered by the small study sizes and retrospective natures of the studies. The *P. aeruginosa*-free interval differed between the studies; ranging from a mean of 3 to 12 months.[33-35] Currently, there is no consensus about the optimal eradication regimen and failure rates of this approach have been estimated around 15-30%, remaining the patient at further risk for developing chronic *P. aeruginosa* infection.[36] Even when apparently eradicated, first *P. aeruginosa* acquisition in young children was being associated with further deterioration in lung function. This suggests that more rigorous strategies to prevent *P. aeruginosa* acquisition are needed.[11] In a Belgium centre, continuous prophylactic inhaled anti-*Pseudomonas* therapy was implemented for patients with high risk of acquiring *P. aeruginosa* infection. Retrospectively, a low prevalence of initial and chronic *P. aeruginosa* infections was observed.[36] A similar study was performed in Austria, where daily inhaled gentamycin prophylaxis administered to young CF children with certain risk factors to acquire *P. aeruginosa* infection showed significant differences in acquisition of chronic *P. aeruginosa* infection.

Since there is a window of opportunity for possible eradication of initial non-adapted, highly susceptible *P. aeruginosa*, it is relevant to detect first *P. aeruginosa* infection as early as possible.[8] This knowledge has led to more frequent culturing of respiratory specimens (sputa, cough swabs, oropharyngeal swabs or broncho-alveolar lavage fluid).

Modulation of virulence factors and inflammation

Macrolide antibiotics such as azithromycin have been shown to suppress inflammation, to interfere with *P. aeruginosa* virulence and to decrease biofilm formation, unrelated to direct antimicrobial effects.[37;38] In vitro- and mouse models showed suppression of quorum-sensing regulated virulence factors by macrolide antibiotics. This significantly improved the clearance of *P. aeruginosa* alginate biofilms and reduced the severity of the lung pathology compared to that in control mice. Also, inhibition of exotoxin production, neutrophil chemotaxis and mucus secretion were potential effects.[16;38] The efficacy of macrolides is related to positive clinical outcomes, namely reduction in lung function decline, increase of weight, and reduction of biofilm-producing strains of *P. aeruginosa* in sputum samples.[39] Placebo-controlled trials showed small benefit with relation to frequency of exacerbations and pulmonary function after 6 to 9 months of macrolide treatment.[40]

Vaccination

Some *P. aeruginosa* vaccines have been successfully tested in preclinical and clinical trials; there is limited evidence for a reduction in *P. aeruginosa* first acquisition and chronic infection. One of the problems with *P. aeruginosa* vaccination trials is the efficacy testing, given the intensive antimicrobial therapy that is administered nowadays after first acquisition of *P. aeruginosa*. Passive vaccination by administration of hyper immunoglobulin derived from healthy donors failed to decrease incidence or severity of *P. aeruginosa* disease.[41;42] *P. aeruginosa* specific monoclonal antibodies are promising, especially in invasive infections in immunocompromised patients, but till now have not been tested in clinical trials with CF patients.[43]

Research questions and outline of this thesis

Because *P. aeruginosa* acquisition is associated with deterioration in lung function, even when apparently eradicated, it is important to prevent *P. aeruginosa* infection of the lung. Early detection of *P. aeruginosa* in the CF airways facilitates immediate eradication treatment after infection. Eradication is probably influenced by the timing of initial treatment, host- and bacterial related factors. The studies described in this thesis address these issues.

Research questions

- What are the bacterial determinants of *P. aeruginosa* acquisition and persistent infection in children with CF?
- What are the diagnostic values of the current detection strategies of initial *P. aeruginosa* infection in children with CF and can these values be improved by measurement

of anti-*Pseudomonas* serum antibodies?
- Can the initial *P. aeruginosa* infection or early colonisation be prevented or delayed by prophylactic 3-monthly courses of inhaled colistin and oral ciprofloxacin?
- What are the side effects of prophylactic antibiotic use in patients with CF?

Outline

In chapter 2, *P. aeruginosa* infections among children with and without CF are described and bacterial genotypes of *P. aeruginosa* causing both CF and non-CF *P. aeruginosa* infections are compared. Chapter 3 focuses on bacterial characteristics of firstly acquired *P. aeruginosa* isolates in CF patients and a comparison is made between the characteristics of eradicated and persistent isolates. In chapter 4, diagnostic values with respect to direct- and indirect detection of *P. aeruginosa* are reviewed. Chapter 5 describes the diagnostic value of serum anti-*Pseudomonas* antibody measurements with respect to early and chronic infection in patients with CF. In chapter 6, a randomised controlled trial into the prevention of initial *P. aeruginosa* infection by 3-monthly cycled antibiotic prophylaxis is analysed. In addition, follow-up data on anti-*Pseudomonas* serology with respect to early infection and the side effects of prophylaxis are evaluated. The long-term effect of maintenance macrolide antibiotic therapy, often prescribed in CF patients with *P. aeruginosa* infections, is addressed in chapter 7. The focus lies on pulmonary function, *S. aureus* colonisation and macrolide resistance rates in *S. aureus*. Data on macrolide-resistant *S. aureus* colonisation in patients with CF and transmission to their household contacts are provided in chapter 8. A summary and critical evaluation of the results described in this thesis can be found in chapter 9, together with recommendations for future research directions.

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Children with Tau Tau puppets - Tana Toraja, Indonesia

2

Pseudomonas aeruginosa diversity in distinct paediatric patient groups

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Abstract

P. aeruginosa is a pathogen that often infects patients that are either immunocompromised or have local defects in host defences. It is known that patients with cystic fibrosis (CF) are sometimes infected with certain clonal isolates. It is not clear whether these clonal isolates also infect non-CF patients and if clonality of isolates occurs in other patient groups. Our aim was to investigate *P. aeruginosa* diversity and the occurrence of clones within 5 distinct paediatric patient groups susceptible for *P. aeruginosa* infection.

P. aeruginosa was cultured from 157 patients (CF 1st infection (29); CF chronic infection (27); urinary tract infection (34); chronic suppurative otitis media (43) and intensive care hospitalization/immunodeficiency (24)). All 202 phenotypically different isolates were tested for antimicrobial resistance and further typed by PFGE. Simpson's index of diversity was calculated for the 5 groups.

CF chronic patients carried highest number of different *P. aeruginosa* phenotypes and genotypes per culture. Isolates from the CF chronic group were significantly less diverse compared to the other groups. A group of clonal isolates was observed among patients from the CF chronic and CF 1 group. These or different clonal isolates were not encountered among the 3 other patient groups. No characteristic resistance pattern could be identified among isolates from the distinct patient groups and among the clonal isolates

Isolates of the CF chronic group were less diverse than in the other patient groups with *P. aeruginosa* infection; clonal isolates were not encountered in patients without CF. Transmission of clonal CF isolates to other patient groups was not observed.

Introduction

Pseudomonas aeruginosa is a common cause of chronic pulmonary infections in patients with cystic fibrosis (CF). There are several hypotheses concerning the increased susceptibility to *P. aeruginosa* infections in patients with CF. It has been suggested that cells with mutant cystic fibrosis transmembrane regulator (CFTR) protein are not able to bind, internalize and kill *P. aeruginosa*.[1] A decreased volume of the pulmonary periciliary liquid layer due to electrolyte disturbances leads to mucus with increased viscosity and impaired clearance. The anaerobic conditions in the mucus layer upregulate alginate production, and mutations promote a switch from non-mucoid to mucoid phenotypes of *P. aeruginosa* that are difficult to eradicate.[2;3] *P. aeruginosa* is a pathogen that infects patients who are either immunocompromised or have local defects in host defences. Once it has attached to the epithelium, *P. aeruginosa* produces type III secretion products that interfere with cell signal transduction. Tissue destruction and immune response are promoted by extracellular products such as elastase, phospholipase C and exotoxin A.[4;5] In CF patients, unique *P. aeruginosa* isolates responsible for first acute infection are probably picked up from the environment. In contrast, CF patients with chronic *P. aeruginosa* infection often share common bacterial genotypes that are either selected or transmitted among patients, so-called 'epidemic strains' or 'clones'.[6-8] *P. aeruginosa* might adapt to the CF host environment by suppression of virulence factor production to evade host phagocytic defense.[9;10]

As well as patients with CF, several other pediatric patient groups are highly susceptible to *P. aeruginosa* infection. Children with chronic suppurative otitis media (CSOM) have damaged inner ear epithelium as a result from previous episodes of acute otitis media. *P. aeruginosa* adheres to the damaged epithelium and might form a biofilm.[11] The same principle applies to children with anatomical or neurological abnormalities of the urinary tract system. Intermittent urethral catheterization or indwelling catheters may damage the epithelial lining of the urinary tract, and introduce *P. aeruginosa* to the urinary tract, sometimes in a biofilm mode of growth.[12] Chronic or recurrent infections with *P. aeruginosa* occur frequently among these patients, and these bacteria are often derived from a hospital source. In intensive care units (ICUs), *P. aeruginosa* incidentally causes pneumonia or bloodstream infections. *P. aeruginosa* pneumonia in ventilated patients is related to mechanical injury to the respiratory epithelium, and facilitated by micro aspiration of infected oral and gastric contents during intubation.[5] Also primary or secondary immunodeficiency (ID) renders patients highly prone to *P. aeruginosa* infection. Not only is the recruitment of neutrophils a major component of protective host response to *P. aeruginosa*, but T-lymphocyte-mediated immunity also appears to play an important role in host defence against *P. aeruginosa*.[13;14]

It is unclear whether specific clonality and adaptation of *P. aeruginosa* isolates occurs in the above mentioned non-CF patient groups with increased susceptibility for *P. aeruginosa* infection. Several studies have been performed to detect *P. aeruginosa* transmission or clonality within a hospital by genotyping; however, in most of these studies, no large distinct patient groups were compared.[15-17] Some of these studies described clonal outbreaks with multidrug-resistant *P. aeruginosa* in ICUs or urology units.[18] On the other hand, CF research groups investigated isolates from sputa of CF patients to establish *P. aeruginosa* clonality, but most of them did not compare these isolates with other isolates from large, standardised

patient groups.[6-8;19] Therefore, our aim was to investigate the occurrence of *P. aeruginosa* clones within and among 5 distinct paediatric patient groups susceptible to *P. aeruginosa* infection.

Materials and methods

Patients and setting

Five groups of paediatric patients with *P. aeruginosa* infection participated in this study. *P. aeruginosa* infections in these patients groups account for *c.* 80 to 90 % of *P. aeruginosa* infections in our hospital. The first group consisted of 29 CF patients with a first positive respiratory culture for *P. aeruginosa* (CF-1). The second group consisted of 27 CF patients with chronic *P. aeruginosa* infection, defined as having had *P. aeruginosa* in sputa or cough swab cultures for > 50 % of all cultures in the last year, according to Lee's criteria (CF-chronic).[20] Thirty-four patients in the third group experienced recurrent urinary tract infection (UTI) and/or colonisation with *P. aeruginosa* predominantly because of underlying neurological or anatomical abnormalities. The fourth group comprised 43 patients with (CSOM) from whom *P. aeruginosa* was isolated. The last group comprised 24 patients who acquired *P. aeruginosa* infection at the ICU and patients with underlying primary or secondary immunodeficiency (ICU/ID).

All patients had regular follow-up at the Wilhelmina Children's Hospital Utrecht, the Netherlands, a tertiary referral centre. Patients were seen both in the in- and outpatient clinics located in the same building. Some of the patients from the UTI group and the CSOM group participated in a trial with regular collection of culture specimens. Outpatients and inpatients were not separated; however most inpatients stayed in private rooms or small wards, except for the ICU. From 2005, a strict CF segregation policy was introduced. *P. aeruginosa* isolates were retrospectively and prospectively collected routinely, or according to indication, between September 2004 and May 2006. For analysis, the first positive culture specimen available per patient was included.

Cultures and susceptibility testing

Patient specimens (sputa, swabs, catheter urine and respiratory secretions) were cultured onto appropriate selective and non-selective media and examined at 24 and 48 hours for presence of *P. aeruginosa* on the basis of morphological features. *P. aeruginosa* was identified by standard microbiological methods on solid media and by a positive oxidase reaction. Further identification for each morphologically distinct colony (mucoid and non-mucoid) was performed by the BD Phoenix automated system (BD Diagnostic Systems, Sparks, MD, USA), which included susceptibility testing (NMIC/ID-panel). In addition, in all cases susceptibility testing was also performed by disk diffusion using Rosco (Neo-Sensitabs) tablets (Rosco, Taastrup, Denmark). When identification by the Phoenix system was ambiguous, an API 20NE (BioMerieux) was performed; when susceptibility testing delivered ambiguous results, a MIC was determined by Etest (AB Biodisk, Solna, Sweden). Susceptibility testing included aminoglycosides, β-lactams (piperacillin/tazobactam, meropenem and ceftazidime), polymyxins

and fluoroquinolones and results were interpreted according to CLSI breakpoints. Multidrug resistance was defined as resistance to more than one of the antibiotic classes mentioned above, as suggested by Paterson.[18] Single colonies of all phenotypic distinct isolates were stored at -80 ºC until handling.

Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed because of its high discriminatory ability, as described previously.[21;22] Briefly, 2 % melting agarose plugs (Sigma A-4018) containing bacterial suspension in an equal volume were incubated for 24 hours at 56 ºC with 10 µl of proteinase K (50 mg/ml) in 1 ml of EDTA-Sarcosine (ES)-buffer. After washing three times with TRIS, NaCl, $MgCl_2$, dithiotreitol (NE)-buffer with bovine serum albumin (BSA), bacterial DNA was digested for 24 hours at 37 ºC with the restriction enzyme *Spe*I (New England Biolabs, Ipswich, MA, USA). Electrophoresis was performed with agarose (1 %) gel (Seakem Gold) in a clamped homogenous electric field system (Chef DR II system, Bio-Rad Laboratories, Hercules, CA). A Lambda ladder control marker (New England Biolabs) was inserted. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV light with Gel Doc EQ (Bio-Rad laboratories). The macro restriction band patterns were analysed by Quantity One and Fingerprinting software (Bio-Rad), and examined visually. Cluster analysis was carried out using Dice coefficients of pattern similarity with the unweighted pair group using mathematical average (UPGMA) algorithm, with a tolerance limit of 1 % for band migration distance. Isolates were considered probably related when having a pattern with ≥ 80 % homology. A clone was defined as genetically related isolates that are indistinguishable or probable related, as investigated by PFGE.[23]

Statistics

To compare parameter means with 95 % confidence intervals (CI) between distinct patient categories, the independent samples Student's *t*-test was used. All statistical calculations were performed using SPSS version 12.0 (SPSS, Chicago, Ill, USA).
To estimate diversity of *P. aeruginosa* isolates in the five distinct groups, Simpson's diversity index with 95 % CI was calculated using Ridom Epicompare software.[24;25] Calculations were performed taking into account all phenotypically different isolates per group. A score of 1 indicates maximum diversity, and a score of 0 indicates no diversity.

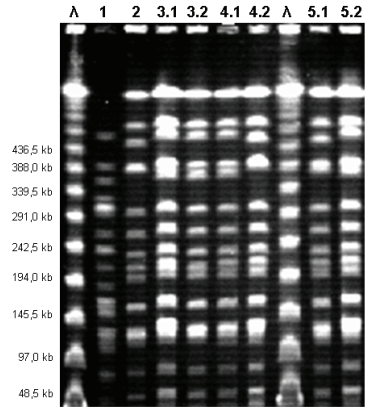
Results

Patients, and P. aeruginosa phenotypes and genotypes

Two hundred and two *P. aeruginosa* isolates from 157 patients were cultured. Table 1 shows the mean number of phenotypically distinct isolates per culture. This number is higher for the CF-chronic group in comparison with all the other groups ($p<0.001$ for all groups). Mucoid isolates were observed only in the CF patients. As the number of genotypes per culture was lower than the number of phenotypes, the phenotypically different isolates did not all have a distinct genotype. The number of genotypes per culture in the CF-chronic group was

higher than that in the other groups (CF-1 (p=0.007), UTI (p=0.079), CSOM (p<0.001) and ICU/ID (p=0.006)).
A group of isolates with ≥ 80 % homology was observed within the CF-chronic and CF-1 groups. Fourteen of 27 patients (52 %) from the CF-chronic group carried such isolates, which were regarded clonal (figure 1 shows some of the isolates with ≥ 80 % homology).

Figure 1 Macro restriction patterns of some clonal isolates



Two patients from the CF-1 group acquired *P. aeruginosa* infection with the clonal genotype. These clonal isolates were not encountered in cultures from patients in the UTI, CSOM and ICU/ID groups. Other genotypically related isolates (≥ 80 % homology) were occasionally encountered in cultures from patients in the other groups (table 1). One patient from the UTI group and one patient from the ICU/ID group carried a related isolate.

Table 1 Patient data and isolate data of the five study groups

	CF-1	CF-CHRONIC	UTI	CSOM	ICU/ID
No. of patients	29	27	34	43	24
Mean age (years) at *P. aeruginosa* isolation for analysis (95 % CI)	7.25 (5.57-8.94)	12.35 (10.71-13.99)	5.77 (3.91-7.63)	6.34 (5.30-7.37)	6.37 (4.20-8.53)
No. of isolates with mucoid phenotype	2	10	-	-	-
No. of phenotypically different isolates	36	56	42	44	24
Mean number of phenotypically different isolates per culture (95 % CI)	1.21 (1.05-1.36)	2.07 (1.66-2.48)	1.24 (1.02-1.45)	1.02 (0.98-1.07)	1.00
Mean number of genotypically different isolates per culture (95 % CI)	1.03 (0.96-1.11)	1.41 (1.13-1.68)	1.15 (0.99-1.29)	1.00	1.00
Patients with related isolates within the group	1 x 2* 1 x 2	1 x 14* 1 x 2	1x2, 1x2 1x2, 1x3	1 x 2	1 x 2

** indicates 2 patients from the CF-1 group that carried clonal isolates and 14 patients from the CF-chronic group with clonal isolates.*

Measurement of P. aeruginosa diversity in each patient group

Simpson's index of diversity (DI) demonstrated that patients from the CF-chronic group carried significantly less diverse isolates (DI 0.90, 95 % CI 0.85-0.95) than patients from the other groups. With respect to diversity, isolates from CF-1 patients (DI 0.99) resembled UTI (DI 0.99), CSOM (DI 1.00) and ICU/ID (DI 1.00) isolates.

In vitro resistance against anti-Pseudomonas antibiotics

Susceptibility testing revealed in vitro resistance against one or more of the tested antibiotics in 14.9 % of isolates (table 2). Twenty-five (12.4 %) of isolates were resistant against one anti-pseudomonas antibiotic, five (2.5 %) isolates were multidrug-resistant, however no isolate was resistant against all evaluated anti-*Pseudomonas* antibiotics. The highest percentage of resistance was found in the UTI group, followed by the CSOM and CF-chronic groups. These resistance rates did not differ significantly from those of the CF-1 and ICU/ID groups (p=0.309). No characteristic resistance pattern could be identified in the distinct patient categories.

Table 2 In-vitro resistance of all *P. aeruginosa* isolates against major anti-*Pseudomonas* antibiotics

	tob	cip	pip	caz	col	tob+cip caz+col	tob+cip +caz	cip+pip +caz	Total resistance
CF-1 (N=36)	1 (3)	1 (3)	-	-	-	1 (3)	-	-	3 (8.3)
CF-chronic (N=56)	2 (4)	-	2 (4)	3 (5)	-	-	2 (4)	-	9 (16.1)
UTI (N=42)	4 (9)	2 (5)	-	2 (5)	-	-	-	2 (5)	10 (23.8)
CSOM (N=44)	-	6 (13)	-	-	-	-	-	-	6 (13.6)
ICU/ID (N=24)	-	2 (8)	-	-	-	-	-	-	2 (8.3)
Total (N=202)	7 (3.5)	11 (5.4)	2 (1.0)	5 (2.5)	-	1 (0.5)	2 (1.0)	2 (1.0)	30 (14.9)

All values are given as no. (%).
tob=tobramycin; cip=ciprofloxacin; pip=piperacillin; caz=ceftazidime; col=colistin

P. aeruginosa in chronically infected CF patients

The fourteen carriers of the CF clone were significantly older than the thirteen CF patients who carried strains of other genotypes (14.52 ± 2.17 *v.* 10.01 ± 4,53; p=0.005). Within the group of thirteen patients with other genotypes, two patients were carrying strains of distinct, probably related, genotypes. Clonal isolate carriers also had infections of longer duration, although not significantly longer (5.69 ± 3.20 *v.* 4.25 ± 3.96; p=0.312). The clonal genotype exhibited several phenotypic expressions. It was observed that in some patients, mucoid and non-mucoid isolates recovered from the same patient belonged to the common clone. In the CF-chronic group, both patients infected with the clone and patients infected with other genotypes carried, on average, more than one phenotype and genotype per culture. Patients carrying the clonal genotype showed higher resistance rates than genotype carriers (22.5 % *v.* 8.0 %), but no uniform resistance pattern was noted.

Discussion

A large amount of diversity in phenotypic expression and resistance patterns of *P. aeruginosa* in five distinct paediatric patient groups (157 patients) with *P. aeruginosa* infection was observed. The majority of *P. aeruginosa* isolates had a unique genotype; however the fact that a group of clonal isolates was detected among CF patients but not in patients with CSOM, UTIs or immunodeficient status, or who were hospitalized in the ICUs, suggests cross-infection, host-specificity or a common source.[6;26] The patients investigated in this study were all prone to *P. aeruginosa* infection because of their underlying conditions. Because clonal isolates were noticed among CF patients, and only occasionally among the others, the factors accounting for this clonality were sought.

First, the aetiology of *P. aeruginosa* infection differs. CF is an intrinsic genetic disorder with loss of CFTR function in epithelial cells. The aetiology for *P. aeruginosa* infection in other patients often involves epithelial damage which increases the possibility of *P. aeruginosa* binding through its pili, and a condition of a continuum between the environment and lungs, inner ear or urinary tract. The specific condition in the CF lung with defective CFTR, electrolyte abnormalities, inflammation and hyperviscous mucus is not comparable with other conditions. Nevertheless, the diversity in the CF-1 group resembles that in the other non-CF patient groups. In CF patients, the first colonisation with unique non-mucoid forms might be eradicated successfully in the early stage; however re-colonisation often occurs with same or different genotype(s). In later stages eradication of the organism is rarely achieved because of hampered penetration of antimicrobials and chronic infection is the consequence. Previous pulmonary infection with unique *P. aeruginosa* isolates might predispose the patient to be colonised by clonal isolates, as does the specific CF lung status. This can be illustrated by the fact that within the CF-1 group, only two of 29 patients showed infection with the clonal genotype. Both patients had mixed infection with another unique genotype. Regarding *P. aeruginosa* infections in the other groups, *P. aeruginosa* may be definitively or temporarily eradicated by antimicrobial therapy; re-introduction might occur because of predisposing and environmental factors. Adaptation of bacteria to the host is possible once eradication is not successful and bacteria stay in the host environment for a longer period.[10]

Second, interpatient contacts may facilitate transmission of bacteria. For CF patients, this has been proven in CF summer camps.[27] However, colonisation with genotypically identical isolates has been documented for CF patients who were separated from each other, suggesting a possible common environmental source.[6] In our hospital, strict CF patient segregation was introduced in 2005. Most of the infections with clonal isolates might have occurred before this segregation. There is evidence that aerosol transmission occurs by coughing; the modes of transmission for CF patients are probably aerogenous, via respiratory secretions or via contaminated (respiratory) equipment or environment, with a high transmission risk in comparison with the transmission risk of patients with other infection sites.[26;28-30] CF patients are not segregated from other patients and transmission of bacteria between CF patients and other patients might occur in the wards, recreational rooms or outpatient clinics via hospital personnel, personal contacts or contaminated environment; however this could not

be proved with the current study groups.
Clonal isolates probably have selective advantages in colonising CF patients. The capacity of *P. aeruginosa* to adapt to its environment might be applicable to the explanations above. Adaptation was examined by scoring development of antimicrobial resistance and mucoidity in the study groups and, as in other studies, different patterns of resistance were noticed among clonal isolates.[6;8] Additionally, antimicrobial resistance in both CF groups was not higher than in the other groups. Mucoidity was observed, but in a minority of isolates in the CF chronic group. Thus, in CF patients another mechanism must be responsible for the high prevalence of clonal genotypes. Clonal CF isolates differ among countries and CF centres (unpublished data); consequently various isolates are able to become a clonal isolate. Down-regulation of virulence factor genes to evade the host response has been observed in these clones as an adaptation strategy; however increased virulence has been proved in other studies.[10;31] Reduced virulence might explain the decreased capacity to infect non-CF patients, as no clonal isolate was observed in the non-CF patient groups. Another survival strategy is hypermutation. Hypermutation is extremely prevalent in cystic fibrosis isolates; however hypermutability seemed not to be more prevalent in clonal isolates.[32;33] Genetic unravelling and performance of virulence factor assays might create understanding of factors necessary to become successful colonisers in CF patients but not in other patients.
The *P. aeruginosa* diversities estimated in this study are based on PFGE typing, which has long been regarded as the reference standard for genotyping. PFGE seems to have even greater discriminatory ability than multilocus sequence typing. However, isolates not designated clonal by PFGE might have common ancestry which can be visualized with multilocus sequence typing.[22;34] Further studies with both older and novel techniques might allow a better understanding of the epidemiology of clones.
Only 2.5 % of the studied *P. aeruginosa* isolates possessed multidrug resistance. In general, resistance against antimicrobials was low, being highest in the UTI group. In a 2005 nationwide surveillance effort, the highest resistance rates were also found among urology isolates, with ciprofloxacin resistance rates of 10 % (Nethmap 2006, Dutch Working Party on Antibiotic Policy). The somewhat (statistically non-significant) higher resistance rates among the CSOM, UTI and CF-chronic groups might be explained by previous antibiotic use. When comparing the present antimicrobial resistance data with those of the worldwide SENTRY antimicrobial surveillance program in paediatric isolates, a two-fold to ten-fold higher percentage of *P. aeruginosa* antimicrobial resistance to ciprofloxacin, ceftazidime, piperacillin, tobramycin and polymyxin was observed in European isolates as compared to our isolates.[35] Low resistance levels might be the result of a strict antibiotic prescription policy in the Netherlands.
A higher percentage (22.5 %) of antimicrobial resistance was noticed among clonal isolates than among other isolates in the CF-chronic group. The patients carrying clonal isolates were significantly older; suggesting the possibility that the cumulative administered dose of antimicrobial therapy was higher. In addition, clonal isolates might posses intrinsic resistance mechanisms unrelated to previous antimicrobial therapy, which facilitate survival since majority of clonal isolates were not antibiotic resistant.

In conclusion, a large diversity in pheno- and genotypes was noted in 202 *P. aeruginosa* isolates from five distinct paediatric patient groups susceptible to *P. aeruginosa* infection. Isolates of the CF-chronic group were significantly less diverse than those of the other groups;

clonal isolates seemed to be restricted to CF patients. Transmission of clonal CF isolates to other patient groups was not observed suggesting that this clone has an as yet unknown selective advantage for colonising CF patients as compared with other patients.

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Adolescent boy working in dyer's souk - Fes, Morocco

Characterisation of eradicated and persistent *P. aeruginosa* isolates from cystic fibrosis patients with early *P. aeruginosa* colonisation

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Submitted

Abstract

Early *P. aeruginosa* acquisition in CF patients is nowadays treated with intensive antimicrobial therapy; in the majority eradication is achieved. Despite intensive therapy, some patients progress rapidly to chronic infection.
Our objective was to assess bacterial characteristics of early *P. aeruginosa* isolates that are either eradicated after one treatment cycle or that persist despite several treatment cycles.

Eighty-six first acquisition episodes from two CF centres were included. First *P. aeruginosa* isolates that were either eradicated or persistent (omitting the intermittent group) were genotyped by SNP-array. Presence of motility, protease and pyocyanin secretion, C4/C12-AHL production, mucoidity, antimicrobial resistance, cytotoxicity on IB3-1 bronchial CF cell-line and mutation frequencies were analysed in-vitro.

Nineteen percent of the patients developed persistent infection; 42 % achieved eradication. The isolates were genotypically highly diverse. Secretion of virulence factors was highly variable among both the eradicated and persistent isolates and did not differ between the groups. Cytotoxicity was present in 57 % of eradicated isolates versus 100 % of persistent isolates ($p=0.002$). Three eradicated isolates showed increased mutation frequencies; none of the isolates were antibiotic-resistant. In-vitro determined bacterial characteristics could not predict persistence after first *P. aeruginosa* colonisation.

Bacterial characteristics of persistent and eradicated isolates were largely variable and did not differ between those groups, except for cytotoxicity. For the individual CF patient, it is therefore not possible to predict the risk of persistence solely on the basis of bacterial characteristics such as virulence, cytotoxicity, antibiotic resistance and mutation frequencies.

Introduction

Abnormal mucociliary clearance, inactivation of antimicrobial peptides by high pulmonary salt concentrations, increased bacterial epithelial adherence and reduced bacterial internalization pose subjects with cystic fibrosis (CF) at risk for chronic pulmonary *Pseudomonas aeruginosa* infection.[1;2] Consequently, early infection is intensively treated with combined antimicrobial therapy leading to, albeit temporal, eradication of *P. aeruginosa* in the majority of patients. Despite, in 10 to 40 % of patients eradication fails, leading to chronic airway infection shortly after the first positive culture.[3-5]

Identification of those patients at risk for progression to chronic *P. aeruginosa* infection is difficult. Host factors predisposing for mucoid *P. aeruginosa* infection are female gender, number of dF508 alleles, decreased lung function and absence of S*taphylococcus aureus* in sputum cultures.[6]

Generally, initial *P. aeruginosa* isolates express multi-factorial virulence.[2] Synthesis of a number of virulence factors such as the type III system components, proteases, pyocyanin and alginate is controlled by the quorum sensing system. This system is regulated by 2 transcriptional activators, *lasR* or *rhlR* which are activated by its autoinducer molecules N-3-oxododecanoyl homoserine lactone (C12-HSL) and N-butyryl-L-homoserine lactone (C4-HSL).[7;8] Progression to chronic infection is characterised by genetic diversification by numerous mutations leading to a gradual loss of virulence, production of biofilm and change in metabolic profiles, leading to various phenotypes.[9;10] *P. aeruginosa* adaptation is initiated early in the infection course and is promoted by the CF airway environment, being inflammatory with anaerobic sputum in the conductive airways.[11]

Uncertainty exists whether in the very early infection phase enhanced virulence or rapid adaptation leading to loss-of-function mutations predispose for persistence of infection despite antimicrobial therapy.[10;12] Type III secretion system enzymes, inducing direct epithelial and neutrophil cytotoxicity, may be associated with persistent infection.[13-15] In non-CF patients suffering from *P. aeruginosa* ventilator-associated pneumonia high type III secretion determined risk for persistent infection despite antimicrobial therapy.[16] Additionally, biofilm production is a strong predictor of chronic infection. In a recently published follow-up study, eradication of first *P. aeruginosa* isolates was higher for non-mucoid isolates (77 %) than for mucoid isolates (50 %).[17] However, a mucoid isolate is not frequently detected as an initial isolate and might possibly be considered as an already adapted isolate to the CF airways, despite being the first isolate to discover.

CF specific highly transmissible *P. aeruginosa* clones are able to chronically infect *P. aeruginosa*-negative patients as well as replace other *P. aeruginosa* isolates in the CF lungs. The success of these clones in promoting persistent infection is apparently related to either high virulence or involvement of an optimized metabolic profile in the absence of high virulence.[18-20] Murine *P. aeruginosa* pulmonary infection models are contradictory with respect to the capacity of non-virulent isolates to persist after initial colonisation.[12;21-23]

To elucidate bacterial mechanisms that may promote persistence, we explored in-vitro characteristics of initial colonising *P. aeruginosa* isolates. Phenotype, genotype, cytotoxicity and transcriptomic profiles were evaluated in isolates derived from CF patients with evident

eradication and from CF patients with evident persistent infection after initial colonisation.

Methods

Patients, samples and microbiology

This study was done in collaboration with two CF centres: the CF centre of the University Medical Centre Utrecht, the Netherlands and the CF centre of the Rigshospitalet Copenhagen, Denmark. All Danish CF patients are seen once a month; Dutch CF patients are seen 3-monthly in the outpatient clinic. At each visit, patients provided a sputum sample; cough swabs or nasolaryngeal suction specimens were obtained if patients were unable to produce sputum. In the Dutch CF centre, patients were fully segregated; in the Danish CF centre segregation was based on microbial cultures.[24] Patient data were extracted from CF databases.

P. aeruginosa was identified by standard microbiological methods on solid media, by a positive oxidase reaction and by C-390 (9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan) resistance (Rosco, Taastrup, Denmark). Susceptibility testing was performed by disk diffusion using Rosco (Neo-Sensitabs) tablets (Rosco, Taastrup, Denmark). Susceptibility testing included aminoglycosides, β-lactams, polymyxins and fluoroquinolones and results were interpreted according to CLSI breakpoints. *P. aeruginosa* isolates were stored at minus 80°C till handling. The initial colonising isolates were collected between 2004 and 2008.

Infection status and included isolates

A *P. aeruginosa* isolate was considered 'initial' when it was the first *P. aeruginosa* ever detected in a CF patient ('never *P. aeruginosa*') or the first isolate detected after at least 1 year of *P. aeruginosa*-free cultures and no maintenance anti-*Pseudomonas* therapy ('*P. aeruginosa* free'). After initial colonisation with *P. aeruginosa*, the majority of patients were treated with antimicrobial eradication therapy. The treatment type differed between the 2 centres, being mainly 3 weeks oral ciproxin plus 3 months inhaled colistin for Danish patients or 3 weeks oral ciproxin plus 2 months inhaled tobramycin for Dutch patients. Patients were categorised as 'eradicated' (with no *P. aeruginosa* returning in cultures within 1 year after one eradication treatment cycle) or as 'persistent' (no eradication achieved despite multiple eradication therapies). To study isolates in clearly different groups, isolates which could not be attributed to one of these categories (intermittent colonisation) were not included in the study. Phenotypic different isolates from one patient (e.g. mucoid and non-mucoid) were both included in the analysis.

Genotyping

Genotyping was performed for all the initial and if applicable consecutive *P. aeruginosa* isolates with a single nucleotide polymorphism (SNP) array comprising 64 markers (Clondiag, Jena, Germany).[25] A hexadecimal code for the genotypic patterns was assigned; using 16 binary SNP codes from the core genome, flagellin and exoenzyme S/U genes. The related-

ness of isolates was estimated by eBurst algorithm (www.eburst.mlst.net). The distribution of alleles from the core- and accessory genome was compared for both groups. Presence of genomic islands and/or pathogenicity factors represented on the SNP-array was estimated by existence of one or more alleles that represent a part of the island.

Phenotyping

Phenotypic measurements were performed on all initial *P. aeruginosa* isolates, in triplicate. Bacteria were grown overnight on Luria-Bertani (LB) agar plates. Swimming motility assay was performed on 0.3 % agarose plates containing AB minimal medium supplemented with glucose (0.5 %) and casaminoacids (0.5 %). One bacterial colony was inoculated to the agar by sterile toothpick. The plates were incubated for 24 hours at 30˚C. Swimming ability was assessed by measuring the maximum diameter of the zone of growth. Twitching motility was performed in 1.3 % agarose plates containing AB minimal medium supplemented with glucose (0.5 %) and casaminoacids (0.5 %). Isolates were stab inoculated with a sharp toothpick into the bottom of the dish. After incubation at 37˚C for 48 hours, the halo zone of growth between the agar and the bottom dish surface was measured.

Protease production in skim milk was measured by inoculating one colony forming unit on a LB agar plate with 10 % skim milk concentration. Zones of protease production were measured after 48 hours of incubation at 37˚C.

For pyocyanin measurement, bacteria were inoculated in 10 ml LB medium and grown for 24 hours. After measuring the optical density at 600 nm (OD_{600}) the cell culture was centrifuged. Five ml of bacterial supernatant was added to 3 ml chloroform ($CHCl_3$) to extract pyocyanin from the supernatant. After one hour the chloroform phase was added to 1 ml 0.2 M hydrogen chloride (HCl) for pyocyanin extraction in acid solution. Pyocyanin production was quantified by measuring the absorbance at an OD_{520} of the acid solution. Concentrations, expressed as micrograms of pyocyanin produced per ml of culture supernatant, were determined by multiplying the OD_{520} by 17.072.[10;26]

Quorum-sensing (C4-HSL and C12-HSL) molecule identification

Acyl-homoserinelactone production was estimated by high pressure liquid chromatography (HPLC) and mass spectrometry (MS). Bacterial cultures were grown in 10 ml LB medium for 18 hours and biomass was estimated by measuring optical density at 600 nm (OD_{600}) while acyl-homoserinelactone in culture supernatant were analysed by HPLC-MS/MS.

HPLC-MS/MS was performed on an Agilent (Torrance, CA) 1100 HPLC system controlled by MassLynx V4.1. Samples were separated on a Gemini C6-phenyl 3µm, 2-mm-ID, 50-mm column (Phenomenex,Torrance, CA), using a flow rate of 0.300 ml/min at 25˚C. A linear water-acetonitrile (ACN) gradient was used, starting at 2 % ACN, going to 40 % CAN in 30 sec, then 100 % ACN in 4.5 min, the 1 min with a flow of 0.500 ml/min, before reverting to the start conditions in 1 min and holding this for 5 min. Both solvents contained 20 mM formic acid. The HPLC was a coupled Quattro Ultima triple mass spectrometer (Waters, Manchester, United Kingdom) with a Z-spray electrospray ionization source using a flow rate of 700 l/h nitrogen at 350˚C; hexapole 1 was held at 30 V, and the cone was held at 25 V. Nitrogen was used as collision gas, and the mass spectrometer operated in positive multiple-reaction monitoring mode (MRM, dwell time 100 ms). MRM were: 0 to 4 min, i) C-4 AHL retention

time (RT) 2.98 min m/z 172 → 102 @ 15eV (quantifier ion) and 172 → 71 @ 15 eV (qualifier) and ii) open lactone-C-4 AHL RT 2.68 min 190 → 120 @25eV (quantifier) and 190 → 71@25eV (qualifier); 4 to 7 min, iii) Oxo-C12 AHL RT 5.08 298 → 102 @ 25V (quantifier) and 298 → 197@25eV (qualifier); and iv) open lactone Oxo-C12 AHL RT 4.67 min 298 → 102@25eV (quantifier) and 298 → 197@25eV (qualifier).
Calibration was done be external standard (in ACN to avoid lactone opening), and response factor for the open form was assumed to be identical to the closed form. Data were processed in Quanlynx 4.1 (Waters) with s/n ratio of 10 (both transitions per compound), giving detection limits in the 10-30 nM range.

Mutation frequency

Inactivation of the anti-mutator genes was determined by measurement of the mutation frequencies after exposure to rifampicin and streptomycin of *P. aeruginosa* isolates in comparison to the reference laboratory strain PA01. Bacterial isolates were grown overnight in 20 ml LB medium, centrifuged at 3000 rpm for 10 min, and resuspended in 1 ml NaCl 0.9 %. One hundred µl of this suspension and serial dilutions were plated on LB plates and LB plates containing 300 µg/ml rifampicin or 500 µg/ml streptomycin. The numbers of colony forming units on all plates were counted after 48 hours incubation at 37°C. The ratio between colonies on LB plate and rifampicin or streptomycin plates was used to calculate mutation rate. An isolate was considered a hypermutator isolate if the mutation frequency after exposure to rifampicin and/or streptomycin was 20 times higher than the mutation frequency of the reference strain PAO1, as published previously. A five- to twenty-fold increase was considered as weak hypermutation.[27;28]

Cytotoxicity

IB3-1 (American Type Culture Collection JHU-52, Manassas, VA) is a compound heterozygote CF bronchial cell line containing a dF508 mutation, and a mutation with a premature termination signal, W1282X. IB3-1 cells were cultivated in Iscove's Modified Dulbecco's Medium (IMDM) containing 10 % fetal calf serum and 0.01 mg/ml gentamycin. For cytotoxicity experiments; 4 x 10^4 cells were seed into a flat-bottom 96-well microtitre plate. After twenty-three hours of incubation at 37°C and 5 % CO_2, cells had grown near confluence and were washed once with sterile phosphate-buffered saline (PBS); 50 µl of antibiotic-free IMDM with 3 % fetal calf serum was added to each well. Bacteria were grown overnight in 10 ml LB medium, washed 2 times in PBS, resuspended in calcium-free PBS, adjusted to absorbance at 600 nm (OD_{600}) of 0.01 and incubated for one hour at 37°C. Fifty µl of bacterial suspension (± 5 x 10^6 bacteria) was co-incubated with the confluent cell-layer for four hours in a 5 % CO_2 incubation chamber. As a control for 100 % lysis, several wells with cell-layer only were mixed with 0.9 % Triton X100. After four hours of incubation; plate was centrifuged at low speed and 50 µl of each supernatant was transferred to a new plate for estimating lactate dehydrogenase release as a measurement for cell lysis (Promega Cytotox 96, Madison, WI). The assay was performed in triplicate for every bacterial isolate on two independent culture occasions. Percentage cytotoxicity was calculated as (mean lysis bacteria - spontaneous lysis) divided by (maximum lysis - spontaneous lysis). In the final calculation, the lysis percentage of 2 separate culture occasions was averaged. Absence of

cytotoxicity was defined as a lysis percentage of < 5 %.

Microarray sample procession and data analysis

Transcriptomic profiles of two eradicated and two persistent *P. aeruginosa* isolates were assessed. These isolates derived from patients with identical CFTR genotype, antibiotic therapy and age. Triplicate experiments were performed for each strain. Bacteria were grown at 37°C in 50 ml of LB medium in a 250 ml Erlenmeijer flask covered with aluminium foil in a shaking incubator, commencing with an OD_{600} of 0.01. Bacteria were harvested in exponential phase at an OD_{600} of 0.5 and stored at -80°C using RNAprotect Bacteria Reagent (Qiagen). RNA was extracted with RNeasy mini kit (Qiagen). Total RNA was transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen). Subsequent cDNA purification, fragmentation and labelling were performed based on prokaryotic sample and array processing protocol from Affymetrix (Santa Clara, CA). The labelled cDNA were then hybridised on Affymetrix *P. aeruginosa* gene chip and stained on GeneChip® Fluidics Station. The probe arrays were finally scanned with GeneChip® Scanner 3000. The raw data (.cel files) were obtained using the Affymetrix GeneChip® Operating System 1.4 (GCOS). Microarray data analysis was performed using bioconductor in R environment (http://www.bioconductor.org). Normalisation and expression index calculation was done with *rma* function.[29] We applied the cut off p-value < 0.05 and fold change > 2 to the data. The fold change is calculated using the average expression levels of all replicates of two strains in each group. The annotations and functional classes were assigned according to the Pseudomonas Genome Database V2 (http://www.pseudomonas.com).

Statistical analysis

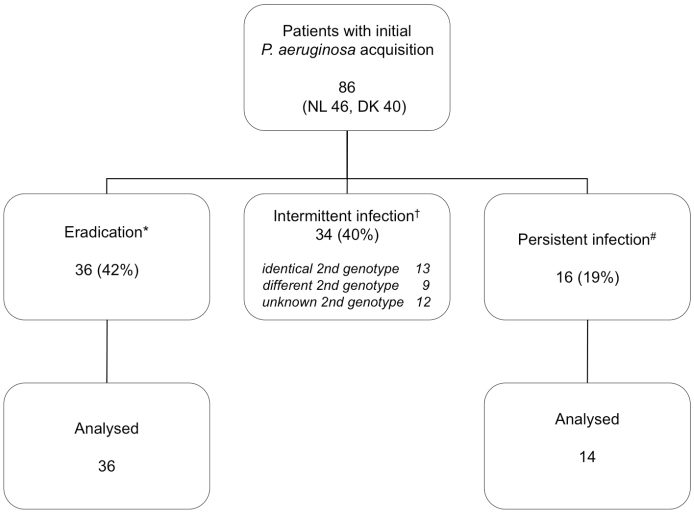
Phenotypic outcomes were divided into categories according to the 15th, 50th and 85th percentiles. Differences in proportions were estimated by χ^2 test or Fisher's exact test. Univariate and multivariate analysis were performed to estimate odds ratios (OR) for bacterial- and patient factors. Factors that showed a p-value < 0.15 were included in multivariate analysis. Continuous data were analysed with Student's *t* test or Mann Whitney U test, depending on normality. Values are expressed as means +/- standard error of the mean (SEM). Measures of association were determined by Pearson or Spearman correlation coefficients. Calculations were performed with SPSS version 15.0 (Illinois, Chicago, USA).

Results

Patients and treatments

Forty-six Dutch- and forty Danish CF patients were included. Mean age of *P. aeruginosa* acquisition was 7.7 year (range 0.8 to 24.1) and was not significantly different between the two CF centres. Females were overrepresented (60 % females *v.* 40 % males), but were on average not younger when acquiring initial *P. aeruginosa* colonisation. Sixteen (19 %) patients progressed to persistent infection shortly after initial *P. aeruginosa* isolation. Thirty-six (42 %) patients achieved long-term eradication. Figure 1 shows the participant flow diagram.

Figure 1 Flow diagram of participants



NL = the Netherlands, DK=Denmark
** > 1 year no P. aeruginosa*
† eradication after more than 1 treatment cycle or eradication after 1 treatment cycle and re-isolation in the year after initial infection
 # no eradication despite several treatment cycles and subsequent genotypes identical

Two patients with persistent infection with the Dutch highly prevalent clone were excluded from further comparative analysis (see below). On average, patients with persistent infection were older than patients with eradication (10.6 versus 5.8 year (p=0.006; OR 1.27; 95 % CI 1.07-1.50)). Previous *P. aeruginosa* isolation did not more often lead to persistent infection. The eradication probability was not different between the 2 centres (p=0.530), the type of eradication therapy was not determinative for eradication success (p=0.450).

Patients with homozygous dF508 mutation did not more often progress to persistent infection and were not significantly younger when progressing to persistent infection. Table 1 shows baseline data of patients with eradication and persistent infection.

Table 1 Patient data

	Eradication (N=36)	Persistence (N=14)	OR (95 % CI)	p-value
P. aeruginosa acquisition age	5.7 ± 0.6	10.6 ± 1.5	1.3 (1.1-1.5)	0.006
Previous single isolation of *P. aeruginosa*	4 (11)	4 (29)	3.3 (0.7-15.6)	0.133
Female gender	23 (63.9)	6 (40)	0.3 (0.1-1.2)	0.097
S. aureus in previous year	24 (71)	9 (64)	0.8 (0.2-2.8)	0.669
H. influenza in previous year	20 (57)	7 (50)	0.8 (0.2-2.6)	0.650
CF centre Utrecht / CF centre Copenhagen	17 (37) / 19 (48)	8 (17)/ 6 (15)	0.7 (0.2-2.3)	0.530
Treatment				
Ciproxin po / colistin inh.	17 (47)	8 (57)		
Ciproxin po / tobramycin inh.	9 (25)	4 (29)		
Monotherapy ciproxin po, inh. colistin or tobramycin.	5 (14)	0 (0)		0.450
Tobramycin/ceftazidim or piperacillin/ tazobactam iv.	1 (3)	2 (14)		
No therapy	4 (11)	0 (0)		
CFTR mutations				
dF508/dF508	21 (58)	10 (71)		
non-homozygous dF508: dF508/other	12 (33)	4 (29)	1.9 (0.5-7.2)	0.342
other/other	3 (8)	0 (0)		
Sputum producers	7 (19)	4 (29)	1.7(0.4-6.9)	0.487

Data are indicated as means ± SEM and N (%)

Genotyping

The isolates were highly diverse in genotype, in both the eradicated and persistent group. Seven genotype pairs were observed; both within the groups and between the groups. The accessory genome of the genotype pairs was distinct except for two genotypes; one type found in both the eradicated and the persistent group and one in the eradicated group. The hexadecimal codes and relatedness can be found in figure 2. Two patients with persistent infections carried the Dutch CF highly prevalent genotype A418.[30] These isolates were omitted from further comparative analysis because of probable distinct transmitting and infecting potential. Fifty-two percent of isolates were exoS positive, 10 % of isolates carried the exoU gene. The exoS gene was not more often encountered in persistent isolates (OR 1.12; 95 % CI 0.33-3.84; p=0.860). Pyoverdin A receptor genes were present in 88 % of isolates. Seventy-six percent of isolates possessed components of the flagellin island; 74 % pKLC-island; 64 % PAGI 2-island. There were no differences in distribution of alleles of the core- and accessory genome and in distribution of one or more genomic islands between the eradicated and persistent isolates.

Figure 2 eBurst tree and genotypes (suffix era=eradicated; suffix per=persistent)

Phenotyping

I. virulence factors

Pili, flagella, protease, pyocyanin, C4-HSL and C12-HSL expression were highly variable both within the eradicated and persistent group. Mean and median expression of these virulence factors was not significantly different between the groups. Phenotype figures and distributions are shown in table 2 and figure 3a. The persistent isolates showed more often motility and protease values ≥ p50; 32 % (eradicated) *v.* 14 % (persistent) had one or more virulence factors < p15. Twitching and swimming motility were correlated (Spearman *r* 0.298; p=0.033); only twitching was correlated with protease (Spearman *r* 0.403; p=0.003). Pyocyanin secretion was not related to any of the other virulence factors. C4-HSL was correlated with protease (Spearman *r* 0.309; p=0.026) but not with the other virulence factors and cytotoxicity. C12-HSL was not related with any of the virulence factors.

Mucoidity was observed in two of the eradicated isolates; all isolates were fully susceptible to tested antibiotics, according to CLSI guidelines.

II. mutation frequencies

Eight percent of eradicated but none of the persistent isolates possessed > 20-fold increased mutation frequency. A > 5-fold increase in mutation frequency (including > 20-fold) was observed in 16 % of all tested initial colonising isolates. The mutation frequencies are noted in table 2. Increased mutation frequency as measured on rifampicin plates occurred only in conjunction with increased mutation frequency on streptomycin plates. Isolates with a > 20-fold mutation rate showed decreased swimming and twitching capacities (p=0.022

and 0.002) and lower protease secretion (p=0.047); pyocyanin secretion was not significantly decreased. More than 5-fold mutation frequency increase was associated with a decrease in cytotoxicity (22.9 ± 4.79 % versus 8.21 ± 1.92 %, p=0.006).

Table 2 Phenotype figures of eradicated and persistent isolates

	Eradication (37)	Persistence (14)	OR (95 % CI)	p-value
Swimming motility (range 0-64 mm)	37.2 ± 2.4	43.56 ± 3.0	1.04 (0.98-1.10)	0.159
N < p15 (29 mm)	7 (19)	0 (0)		0.168
N ≥ p50 (41 mm)	20 (54)	8 (62)		0.843
N > p85 (50 mm)	3 (8)	5 (39)		0.021
Twitching motility (range 0-41mm)	25.4 ± 2.3	29.8 ± 3.4	1.03 (0.98-1.08)	0.308
N < p15 (2 mm)	7 (19)	1 (7)		0.407
N ≥ p50 (31 mm)	17 (46)	10 (71)		0.104
N > p85 (38 mm)	5 (14)	4 (29)		0.236
Protease (range 0-29mm)	23.8 ± 1.0	26.0 ± 0.8	1.14 (0.93-1.40)	0.204
N < p15 (21 mm)	7 (19)	1 (7)		0.419
N ≥ p50 (26 mm)	16 (43)	12 (79)		0.024
N > p85 (28 mm)	10 (27)	3(21)		0.733
Pyocyanin (range 0.10-24 µg/ml)	8.5 ± 1.1	7.5 ± 1.5	0.97 (0.88-1.08)	0.622
N < p15 (0.42 µg/ml)	4 (11)	3 (21)		0.376
N ≥ p50 (7.50 µg/ml)	19 (51)	7(50)		0.931
N > p85 (15 µg/ml)	6 (16)	1(7)		0.657
One or more virulence factors (motility, protease, pyocyanin) ≥ p50	30 (81)	14 (100)		0.169
One or more virulence factors (motility, protease) ≥ p50	26 (70)	14 (100)		0.023
C4-HSL (range 0-15.17 µM)	7.28 ± 0.63	8.04 ± 0.76	1.06 (0.89-1.27)	0.496
C12-HSL (range 0-1156 nM)	188.74 ± 23.56	301.57 ± 340.62	1.00 (0.99-1.01)	0.128
Streptomycin mutation frequency	7.40 ± 4.41*10^{-8}	1.09 ± 0.92*10^{-8}	0.96 (0.84-1.01)	0.563
Rifampicin mutation frequency	30.29 ± 23.14*10^{-8}	1.36 ± 0.13*10^{-8}	0.54 (0.22-1.32)	0.175
Cytotoxicity	17.8 ± 4.4	28.8 ± 8.3	1.01 (0.99-1.03)	0.226

Data are indicated as mean ± SE or N (%)

III. cytotoxicity IB3-1 CF bronchial cell line

Most isolates displayed moderate or limited cytotoxicity with eight isolates showing more than 50 % cytotoxicity. Absence of cytotoxicity, defined as < 5 % lysis, was observed among 43 % of eradicated isolates and 0 % of persistent isolates (p=0.002). Mean cytotoxicity can be found in table 2. Figure 3b shows cytotoxicity for both groups. Cytotoxicity was associated with twitching motility (Spearman *r* 0.378; p=0.006) but not with the other virulence factors. The eight *P. aeruginosa* isolates that induced > 50 % cytotoxicity displayed in general no higher virulence with respect to the other factors; they were not more often encountered in CF patients with homozygous dF508 genotype.

Figure 3a Phenotypic measurements with medians (● eradicated isolates and ▲ persistent isolates)

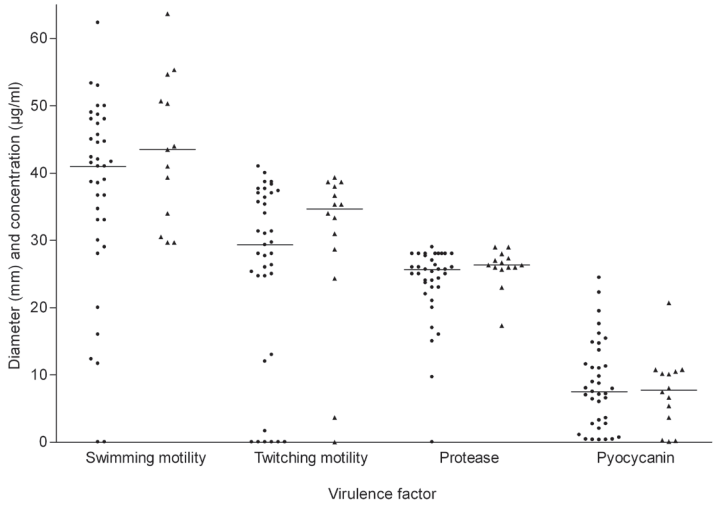
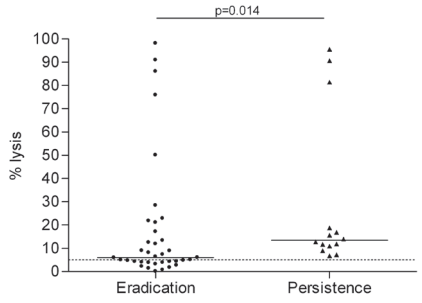


Figure 3b Cytotoxicity (% lysis with medians) on IB3-1 cell layer (dotted line represents 5 % cut off)



Multivariate modelling of in-vitro phenotypic characteristics results revealed no significant bacterial characteristics associated with persistence. Odds ratios with confidence intervals and p-values are displayed in table 2.

Transcriptomic profiles

Assessment of other factors important for the early establishment of persistent *P. aeruginosa* infection was performed by transcriptome analysis using Affymetrix GeneChip based on PAO1. Two eradicated and two persistent isolates from four different patients were included. These isolates derived from patients with a first acquisition age between 5.67 and 6.81 years old. They all received ciproxin and tobramycin eradication treatment and all had CFTR homozygous dF508 genotype.

To compare the transcriptomic differences between the eradicated and persistent isolates, a loggit-*t* test comparing the expression data of the two groups was done. Seventeen genes (table 3) were differently expressed between the eradicated group and persistent group. Seven genes showed increased expression in the persistent isolates compared to the eradicated isolates. Four of these genes (*dctA*, PA5167, PA5168 and PA5169) belong to C_4-dicarboxylate transport system.

Table 3 Differentially expressed genes comparing persistent isolates and eradicated isolates

Gene	p-value	Fold change	Product name	Functional classes
Up regulated in persistence group				
dctA	0.003	3.59	C4-dicarboxylate transport protein	Transport of small molecules
PA1711	0.030	2.39	ExsE	Hypothetical, unclassified, unknown, Transcriptional regulators; Protein secretion/export apparatus
PA2252	0.046	2.48	probable AGCS sodium/alanine/glycine symporter	Transport of small molecules
PA5167	0.010	2.74	probable c4-dicarboxylate-binding protein	Membrane proteins; Transport of small molecules
PA5168	0.007	2.29	probable dicarboxylate transporter	Membrane proteins; Transport of small molecules
PA5169	0.012	2.12	probable C4-dicarboxylate transporter	Membrane proteins; Transport of small molecules
tRNA_Phe	0.030	2.71		
Down regulated in persistence group				
PA0612	0.011	- 4.06	repressor, PtrB	Transcriptional regulators
PA0613	0.024	- 3.61	hypothetical protein	Hypothetical, unclassified, unknown
PA0633	0.018	- 3.44	hypothetical protein	Related to phage, transposon, or plasmid
PA0635	0.035	- 2.48	hypothetical protein	Related to phage, transposon, or plasmid
PA0636	0.030	- 2.48	hypothetical protein	Related to phage, transposon, or plasmid
PA0637	0.023	- 2.42	conserved hypothetical protein	Related to phage, transposon, or plasmid
PA0638	0.027	- 2.24	probable bacteriophage protein	Related to phage, transposon, or plasmid
PA0639	0.017	- 2.20	conserved hypothetical protein	Related to phage, transposon, or plasmid
PA1096	0.042	- 2.09	hypothetical protein	Hypothetical, unclassified, unknown
tRNA_Arg	0.022	- 2.08		

Discussion

This is the first study to describe characteristics of a large sample of early *P. aeruginosa* isolates from patients with CF who progressed either to persistent infection or in whom eradication was achieved. It is important to gain more understanding of the initial infection process to improve treatment of initial *P. aeruginosa* acquisition in order to subsequently prevent chronic *P. aeruginosa* pulmonary infection.
While chronic persistent infection is in general characterised by bacterial adaptation and attenuation of virulence, the results of this study show that the early onset of persistent infection is not associated with in-vitro determined bacterial characteristics.

It can be debated whether the isolates in our study actually resided for only a limited period within the CF airways. Because of the, albeit small, time span between routinely performed respiratory cultures and sampling technique errors, it is impossible to estimate the colonisation time. There were no differences in persistence between the Dutch and Danish centres despite more regular culturing within the Danish centre. The distinction between eradicated and intermittent isolates is problematic because in majority of patients, *P. aeruginosa* infection is a continuous process, with *P. aeruginosa* intermittently isolated for a long period of time before chronically settling. Upper airway colonisation, such as sinus colonisation, might lead to intermittent isolation from the lower airways.[31] Therefore, we categorised patients only as eradicated when there were enough follow-up cultures for a period of at least one year. Additionally, the differentiation between *P. aeruginosa* colonisation and infection is gradual and uncertain.[22;32] The majority of patients with CF does not have respiratory symptoms when *P. aeruginosa* is detected for the first time while airway inflammation as determined by broncho-alveolar lavage studies is present.[17;33]

Our results show a large variability in bacterial characteristics between isolates. While there is a trend towards a somewhat higher virulence in persistent isolates, prediction for the individual patient to progress to persistent infection based on bacterial virulence, mutation frequency and cytotoxicity is impossible. Hypermutation was strongly associated with decreased expression of virulence factors and cytotoxicity. The fact that some identical bacterial SNP genotypes were observed both among the eradicated and persistent isolates, and that the two groups did not diverge from each other in transcriptomic data, indicates that the genomic background of a certain strain is not decisive on whether it can persist in the CF lung. However there are some traits which might be essential in this process. Seven genes showed increased expression in the persistent isolates compared to the eradicated isolates. Four (*dctA*, PA5167, PA5168 and PA5169) of them belong to C_4-dicarboxylate transport (dct) system. The metabolism of C_4-dicarboxylates, such as fumarate, malate, succinate and L-aspartate, requires a bacterial uptake system. In *E. coli*, DctA, a cation (H^+ or Na^+): C C_4-dicarboxylate symporter, is the major carrier in aerobic growth.[34] *DctA* mutants show very poor growth on C_4-dicarboxylates. In *Pseudomonas chlororaphis* O6, *DctA* is also required for C_4-dicarboxylates utilisation and effective root colonisation.[35] PA5167-69 are homologous to *dctPQM*, a tripartite ATP-independent periplasmic transporter with high affin-

ity to C_4-dicarboxylates.[36] In *P. aeruginosa*, there are four homologous operons of *dctPQM* including PA5167-69 and their function has not been studied. In conclusion, it is likely the *dctA* and *dctPQM* genes are responsible or partly involved in C_4-dicarboxylates uptake in certain conditions. Upregulation of those genes in the persistent isolates indicate that the C_4-dicarboxylates transport might be important for early infection of *P. aeruginosa*. It could also give hints on carbon sources that *P. aeruginosa* depends on in the early CF infection. However, expression of *dct* genes has to be examined in a bigger sample size to prove their importance.

Most *P. aeruginosa* isolates were limited or moderately cytotoxic on the IB3-1 cell-line. Using a cut-off point of 5 % for the presence of cytotoxicity; 69 % of *P. aeruginosa* isolates exhibited cytotoxicity, with significantly more cytotoxic isolates in the persistent group. Increasing the co-incubation time and adding of EGTA, a calcium chelator, did not lead to major changes in cytotoxicity. We did not measure type III secretion proteins, as the relation between type III secretion proteins and cytotoxicity has been extensively studied before.[37] Interestingly the percentage of cytotoxicity in our study group corresponds to the data of Jain et al, showing around 50 % of early CF *P. aeruginosa* isolates expressing type III secreting proteins.[14-16] Twitching motility was strongly associated with cytotoxicity, confirming the requirement of pili-mediated bacterial-epithelial contact.[1]
El Solh et al. investigated neutrophil cytotoxicity of *P. aeruginosa* derived from patients with ventilator associated pneumonia and observed a higher cytotoxicity in persistent isolates. However, the variance was not shown. Although the difference in mean cytotoxicity was statistically significant in our study, there was a large variability in both groups and the odds ratio was close to one. For a subset of 18 isolates from our study, human neutrophil cytotoxicity was assessed. The neutrophil cytotoxicity was correlated with IB3-1 cytotoxicity (Pearson r 0.700; p=0.002). In an injured lung, CF host features are probably more important in the infection process than bacterial characteristics, unlike in non-CF patients.
Our in-vitro experiments do not reflect the in-vivo situation in the CF airways but rather the bacterial ability to display a set of chosen factors in in-vitro selected artificial conditions. It could be that the persistent isolates differ in other, yet unknown factors. However the host-pathogen interaction, leading to up-or down regulation of virulence components, is apparently determinative for persistence of infection. This might be illustrated by the fact that a few patients shared identical bacterial genotypes. These genotypes were eradicated in some patients and persistent in the others. In addition to the host-pathogen interaction, co-colonisation with other microbial organisms is significant for up- and/or down regulation of important virulence genes.[38] For instance, viral infections lead to increased *P. aeruginosa* epithelial adherence and renewal of virulence.[39] In this study, we did not estimate viral colonisation during or previous to *P. aeruginosa* acquisition. Communication between *P. aeruginosa* and oral microflora might lead to upregulation of virulence factor genes, consequently promoting invasive infection and possible persistence.

Most isolates excreted virulence factors in-vitro. These findings lead to the discussion whether in addition to intensive antibiotic therapy; treatment directed at virulence should be initiated to reduce the risk for persistent infection. Macrolide antibiotics such as azithromycin reduce motility, inhibit the quorum sensing system and impair oxidative stress response.[40]

Nevertheless, upregulation of type III secretion system was observed in-vitro, leading to the opposite effect.[41] Importantly, virulence factors are a major target for the host immunity and are necessary for clearance of bacteria. Therefore, quorum sensing inhibiting strategies need to be tested in-vivo to ascertain the potential for preventing persistent infection in the early phase. Active or passive vaccination with vaccines containing *P. aeruginosa* virulence factors components such as the type III system component *PcrV*, pili or flagella are promising but only preclinical studies have been executed so far. [42] Reduction of oxidative stress by inhalation of reduced glutathione, led to clearance of *P. aeruginosa,* even in patients with chronic infection.[43]

In conclusion, in-vitro bacterial characteristics of initial *P. aeruginosa* isolates largely vary. Although cytotoxicity is on average higher among the persistent isolates, it is impossible to predict the chance of persistence solely on bacterial characteristics, rendering yet unknown host factors determinative. Intensive eradication therapy for all patients with first *P. aeruginosa* colonisation remains advised and in the future, it should be investigated whether adjuvant therapy targeted at specific bacterial factors to prevent persistent infection with *P. aeruginosa* shows benefit.

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Palestinian boy - East Jerusalem, Israel

Detection of *Pseudomonas aeruginosa* in patients with cystic fibrosis

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Abstract

Chronic pulmonary infection with *Pseudomonas aeruginosa* in patients with CF is associated with a high morbidity and mortality. Adequate treatment of first acquisition of *P. aeruginosa* might prevent or postpone chronic infection. Early detection of *P. aeruginosa* is therefore of major importance. Currently, oropharyngeal cultures or sputum cultures are most commonly performed. However, the oropharyngeal culture has limitations in both the positive and negative predictive value for the presence of *P. aeruginosa* in the lower respiratory tract. Induction of sputum has little additional benefit in detection of *P. aeruginosa* by culture. Molecular techniques are not yet widespread used for detection of *P. aeruginosa* from respiratory samples. They have good diagnostic values, depending on the primer. Serological tests are sensitive for detection of chronic infection but their contribution in detection of early infection and treatment is unclear. In this review, the different diagnostic techniques for detection of *P. aeruginosa* are evaluated.

Introduction

Cystic fibrosis is an inherited multi-system disease, caused by a mutation in the CFTR-gene. The disease is characterised by dysfunction of the exocrine glands and subsequent chronic obstruction in lungs and exocrine pancreatic insufficiency. Chronic obstruction in the lung predisposes for pulmonary infection. Hyperinflammation by persistent infiltration of neutrophils in the airways, even in patients without clinical apparent disease, precedes and accompanies pulmonary infection.[1] In infants, pulmonary infections with *Staphylococcus aureus* and *Haemophilus influenzae* are common. Later in childhood, infections with *Pseudomonas aeruginosa* become dominant. Twenty percent of one-year old infants are infected with *P. aeruginosa,* with an increase in incidence of about thirty-three percent in three-year olds till eighty percent in adulthood.[2] Infection with *P. aeruginosa* is a multi-stage process. Initial infection is characterised by adhesion of *P. aeruginosa* to the epithelial lining of the respiratory tract, and may be transient. If not, aggressive antimicrobial treatment may eradicate *P. aeruginosa* in this early stage and postpone chronic colonisation.[3-8] Eventually, intermittent colonisation becomes chronic and phenotypes of *P. aeruginosa* change to biofilm producing antibiotic-resistant strains.[9] Chronic *P. aeruginosa* infection is associated with deterioration of lung function and disease progression, mediated by host inflammatory, mainly neutrophilic, response.[10;11]

Standard methods for assessing respiratory infections with *P. aeruginosa* are bacterial cultures of sputum, oropharyngeal- or cough swabs or broncho-alveolar lavage fluid (BALF). Culture of BALF can be defined as the reference standard, because it reflects colonisation of the lower respiratory tract. However, BAL is an invasive procedure, and therefore not routinely performed as a diagnostic screening test. Culturing sputum or oropharyngeal swabs is nowadays the most frequently used method to establish *P. aeruginosa* carrier status. Molecular identification techniques to detect *P. aeruginosa* in respiratory specimens are performed increasingly.[12-14] Additional detection of *P. aeruginosa* infection with serological methods before identification by culture might be promising, especially in young patients.[11;15;16]

In this review, we evaluate the clinical values of direct and indirect techniques for detection of *P. aeruginosa* infection in patients with CF. Table 1 summarises the techniques that are available.

Direct detection

Culture

P. aeruginosa, a gram-negative aerobic rod, is easily cultured on different media. On blood agar, a non-selective medium, *P. aeruginosa* is sometimes overgrown by oropharyngeal flora. Gram-negative selective media like McConkey agar make discrimination of *Pseudomonas* species from other respiratory pathogens and indigenous flora more convenient. The oxidase test distinguishes *Pseudomonas* species, which are oxidase positive, from other gram-negative organisms. Further identification of bacterial colonies can be performed by assessing enzymatic reactions, fermentation and susceptibility by *eg.* Phoenix or VITEK automated systems

or API manual system. Selective agents or plates like *Pseudomonas* isolation (irgasan) agar, cetrimide agar and C-390 (9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan) agar or tablets have been especially developed for growth of *P. aeruginosa* while inhibiting other closely related species.[13;17;18].

Molecular techniques and phenotyping

Molecular techniques have in general a high sensitivity for detection of *P. aeruginosa* in sputum in culture-positive patients. Karpati et al demonstrated presence of *P. aeruginosa* in sputum by polymerase chain reaction (PCR)-technique using a primer based on 16S rRNA sequences and compared them with cultures (reference standard). A sensitivity of 93 % and specificity of 90 % was found.[19] Other studies showed sensitivities between 97 % and 100 %, depending on primers.[12;13;20] For example, PCR with outer membrane protein (oprL) primer is more sensitive than exotoxin A (ETA) primer.[14] Molecular detection of *P. aeruginosa* in sputum might be a useful technique with respect to early detection. In ten culture-negative patients with a positive PCR, culture became positive subsequently in five patients within a mean time of 4.5 months.[13] In a different study, many PCR-positive culture-negative samples were found. Eighty three percent of these samples were oropharyngeal samples. A sampling error in oropharyngeal cultures or misidentification was not ruled out and no follow up was done.[12] Fluorescent *in situ* hybridisation (FISH) is based on fluorescently-labeled oligonucleotide probes that target and hybridise rRNA in sputum, which can be visualised by fluorescence microscopy. Sensitivity of FISH in comparison with culture is 80-90 %, depending on methods, and it has a high specificity (100 %).[21;22]
Rapid species identification by analysis of a characteristic protein composition (mass spectrometry), which can be compared with a reference database, is a new technology allowing specific identifation.[23]

The nature of the respiratory specimen plays an important role in detection of *P. aeruginosa*. Below, we describe the value of cultures from the upper respiratory tract and cultures of induced sputum in relation to culture of BALF or spontaneously expectorated sputum.

Naso- or oropharynx

In young children, oropharyngeal swab cultures are often used to identify respiratory pathogens. This also counts for older patients who are not able to expectorate sputum. The diagnostic accuracy of oropharyngeal culture has important clinical implications because till now, it is the only non-invasive way of assessing respiratory tract pathogens.[17] Table 2 shows diagnostic and predictive values of oropharyngeal cultures compared with BALF-cultures. In young asymptomatic children, *P. aeruginosa* positive oropharyngeal cultures do not reliably predict the presence of bacterial pathogens in the lower airways, however negative cultures indicate that *P. aeruginosa* is unlikely to be present in the lower airways.[11;17;24]

Table 1 Available methods for direct- and indirect screening of *Pseudomonas aeruginosa* infection in CF patients

Methods	Examples
Culture	
Processing	
Culture media	Blood agar
	McConkey agar
	Pseudomonas isolation (Irgasan) or Cetrimide agar
Identification	Phoenix, VITEK, API systems
	C-390 resistance
Site	
Nasopharynx	Nasopharyngeal aspirate/swab
Oropharynx	Oropharyngeal swab
	Cough swab
Bronchi	Sputum spontaneously expectorated
	Sputum induced by hypertonic saline
Bronchi/alveoli	Broncho-alveolar lavage (BAL)-aspirate
Geno- or phenotype characterisation	
PCR	16S rRNA gene sequencing
	P. aeruginosa PCR with specific primer
FISH	Fluorescent in situ rRNA hybridisation
Mass spectrometry	Matrix-assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)
Serum antibody tests	
ELISA	Non-purified antigens e.g. whole cell protein
	Purified antigens e.g. cell surface antigens
Western Immunoblot	Non-purified antigens
	Purified antigens
Crossed Immune Electrophoresis	Precipitins with different antigens

In older children and symptomatic patients, there is a higher prevalence of *P. aeruginosa* in oropharyngeal cultures with a concomitant better positive predictive value.[25;26] Combining results of consecutive oropharyngeal cultures reduces the number of false negative cultures, and therefore increase negative predictive value.[11] In patients with *P. aeruginosa* both cultured from BALF and oropharynx, Pulsed Field Gel Electrophoresis (PFGE) showed poor predictive accuracy of oropharyngeal isolates for lower airway organisms.[24] This indicated that the actual positive predictive value might be different.

Table 2 Diagnostic values (in %) of oropharyngeal cultures for *Pseudomonas aeruginosa*, with BALF-culture as reference standard (sens=sensitivity, spec=specificity, P/NPV=positive/negative predictive value)

Author	BAL no	Age	Ref. standard	Sens	Spec	PPV	NPV
Ramsey 1991	43	4-300 months	BAL *P. aeruginosa* any growth	46	93	83	70
Armstrong 1996	150	< 52 months	BAL *P. aeruginosa* > 10^5 cfu/ml	71	93	57	96
Rosenfeld 1999	141	< 60 months	BAL *P. aeruginosa* > 10^5 cfu/ml	82	94	64	97
	119	< 18 months		44	95	44	95
	82	> 18 months		68	94	76	91
Burns 2001	108	< 36 months	BAL *P. aeruginosa* any growth				
			1 OP culture			69	85
			2 OP cultures			83	97

Fewer data exist on sensitivity of nasopharyngeal aspirate- and cough swab cultures. Cough swab cultures showed, in a small group of thirty patients, high positive predictive value but low sensitivity in comparison with cultures of spontaneously expectorated sputum.[27] Nasopharyngeal aspirates showed equal *P. aeruginosa* yield in comparison with oropharyngeal cultures in a group of 47 young children, with no extra *P. aeruginosa* detected in the nasopharyngeal aspirate.[28]

Its low predictive value for presence of pathogens in the lower respiratory tract, as indicated by BALF-culture as a reference standard, limits single oropharyngeal cultures as a reliable detection method. Temporary *P. aeruginosa* colonisation possibly explains these low positive predictive values. Errors or differences in sampling technique may also be responsible for low values, although sampling technique was extensively evaluated in cited studies. Sensitivities in table 2 are to be compared with caution, because different definitions were used for a positive BALF-culture, and so definition of the reference standard.

Cultures of sputum

Simultaneous detection of *P. aeruginosa* in sputum and BALF samples is not widely described. In 17 sputum-producing adult CF patients, BALF showed seven times an extra *P. aeruginosa* strain compared with the sputum sample. In two patients, *P. aeruginosa* infection was newly diagnosed by BAL.[29] In an older study, culture of sputum of CF patients showed high correlation with culture of thoracotomy specimens.[30]

Controversy exists whether induction of sputum for detection of *P. aeruginosa* is of benefit in children with CF. Originally developed for measuring inflammatory parameters in induced sputum in asthmatic subjects, recently this technique has also been used for detection of lower respiratory tract pathogens in patients with CF.[31;32] Induction of sputum by inhaling nebulised hypertonic saline is a relatively easy and safe procedure.[31;33;34] The volume of induced sputum is significantly larger than spontaneous expectorated sputum, also in previous non-expectorating children.[33;35;36] It is a reliable procedure as measured by a high reproducibility of inflammatory markers and respiratory pathogens.[35] Nevertheless, it is not frequently used in young children because of inducible bronchoconstriction.

Table 3 shows results of *P. aeruginosa* status before and after sputum induction. In a young patient group with respiratory symptoms, 32 % of forty-one patients demonstrated more and/or other specimens of bacteria after sputum induction.[37] In total, this study group had a low number of *P. aeruginosa* positive patients and only one additional *P. aeruginosa* was found in induced sputum. In other studies with non-symptomatic patients, slight differences were found between cultures before and after sputum induction.[31;34;36;38] In a small study group, Henig found high agreement between cultures of spontaneous expectorated sputum, induced sputum and BALF regarding mucoid *P. aeruginosa*, while there was less agreement for non-mucoid *P. aeruginosa*.[33] Mussaffi described a larger, although not significant, increase in diagnostic yield after sputum induction.[38]

Consequently, it seems that induction of sputum for detecting *P. aeruginosa* has relatively little practical benefit, although existing data regarding *P. aeruginosa* positive cultures after sputum inductions were derived from small study groups.

Important to mention is that most study groups existed of non-symptomatic patients. One reason for low additional detection might be that *P. aeruginosa* infection by itself gives a higher chance of spontaneous sputum production. Differences between culturing BALF and induced sputum are not described clear. However, in comparison with BAL, it is a non-invasive and less intensive procedure.

Table 3 Additional detection of *Pseudomonas aeruginosa* with sputum induction in patients with CF

Author	Sample size	Age (year)	*P. aeruginosa* pre-induction %	*P. aeruginosa* post-induction %
de Boeck 2000	15	4.3-15.2	33	47
Sagel 2001	19	7-12	32	21
Henig 2001	10	19-38	PA non-mucoid 10 PA mucoid 60	30 60
Suri 2003	32	7.3-17	56	59
Ho 2004	41	1.8-12.9	7	7
Mussaffi 2008	29	0.7-8	17	38

Indirect detection

Serum antibody detection

Infection with *P. aeruginosa* can be proven both by culturing of the organism itself and by detection of immune response to the micro-organism. Antibody testing with ELISA demonstrated little or no interference from cross-reacting antibodies directed against other bacteria.[11;16;39]

The purified antibodies against *P. aeruginosa* show different trends in elevation during infection. There is a temporal hierarchy described in development of antibodies, reflecting *P. aeruginosa* virulence mechanisms: *pop*B, exoenzyme S, exotoxin A and phospholipase C antibodies seem to develop first, the time to titre elevation of elastase and alkaline protease antibodies is prolonged. In addition, frequencies of elevated antibodies seem to vary among

different antigens.[16;40-42]

There are several factors influencing antibody development. Mucoid *P. aeruginosa* strains induce a more pronounced antibody response compared to non-mucoid strains.[40;43] Dual colonisation with *Staphylococcus aureus* can be accompanied with lower antibody titres of exotoxine A and elastase, but with higher titres of phospholipase C.[41;44]

Antibody titres rise during periods of active infection. There is a decrease noted with antimicrobial treatment.[41] Chronically *P. aeruginosa* infected CF patients who are treated intensively with antibiotics have lower antibody responses.[7;41;45;46] Continuous steroid treatment is accompanied by a decline in antibody titre.[15]

Theoretically, other foci of *P. aeruginosa* infection can induce an increased antibody titre. Examples are ear infection, wound infection and cystitis. Not much literature exists on development of anti-*P. aeruginosa* antibodies in these infections. Few patients with *P. aeruginosa* cystitis, ear- or wound infection showed antibody response, and in general, this response was lower than the response seen in CF patients with pulmonary *P. aeruginosa* infection.[47]

Most diagnostic antibody tests are based on IgG antibodies. Other serum immunoglobulins, especially IgA, also show elevation of specific *P. aeruginosa* antibody titres. Elevation was particularly seen in patients with pulmonary *P. aeruginosa* infections, other foci elicited fewer specific IgA antibodies.[39;48] In addition, titre of anti-*P. aeruginosa* IgA, like IgG, correlates with disease severity and might be a better parameter for follow-up than IgG, because it reflects endobronchial infection.[48;49]

Antibody response in chronic Pseudomonas aeruginosa infection

Patients with chronic *P. aeruginosa* infection show high (more than 2 standard deviations above those of normal control values) IgG titres to proteins of *P. aeruginosa*.[50;51] Sensitivity and specificity of serology in chronic colonisation differ slightly among distinct methods and antigens. Table 4 shows the sensitivities of diverse antibody tests. Antibody levels obtained by whole cell protein ELISA and level of precipitins correlated with each other and showed both a sensitivity between 96 and 100 % for *P. aeruginosa* colonisation.[52;53] Instead, ELISA or Western Blot with a single purified antigen has a lower sensitivity for confirming chronic colonisation, and is antigen-dependent.[40;54-56] No single antigen was able to detect the antibody response in all CF patients.[41;57]

Table 4 Sensitivity of single sample antibody testing for *Pseudomonas aeruginosa* infection

Author	Sample size	Test	Antigen		Sensitivity %
Chronic colonisation					
Granstrom 1984	22	ELISA	Phospholipase C		100
			Exotoxin A		68
			Alkaline Protease		73
			Elastase		23
Hollsing 1987	26	ELISA	Phospholipase C		100
			Exotoxin A		58
			Alkaline Protease		58
			Elastase		15
Pedersen 1987	91	ELISA	Whole cell protein		96
	108	CIE	64 different antigens		100
Kappler 2006	88	ELISA	Alkaline Protease		75
			Elastase		65
			Exotoxin A		83
			Combination of 3 antigens		98
Tramper-Stranders 2006	67	ELISA	Alkaline Protease		76
			Elastase		87
			Exotoxin A		79
			Combination of 3 antigens		96
Ratjen 2007	164	ELISA	Exotoxin A		72
			Alkaline Protease		85
			Elastase		76
			Combination of 3 antigens		93
Pressler 2009	281	CIE	64 different antigens		96
		ELISA	Whole cell protein		97
		ELISA	Exotoxin A		93
Intermittent colonisation					
Pedersen 1987	20	CIE	64 different antigens		50
Kappler 2006	27	ELISA	Alkaline Protease		26
			Elastase		15
			Exotoxin A		41
			Combination of 3 antigens		48
Tramper-Stranders 2006	60	ELISA	Alkaline Protease		17
			Elastase		42
			Exotoxin A		48
Early colonisation with 1st positive culture					
Brett 1988	33	ELISA	Whole cell protein		88
Burns 2001	18	ELISA	Exotoxin A	BAL +	94
	11			OP only +	91
	18	Immu-noblot	Whole cell protein	BAL +	94
	11			OP only +	82
Ratjen 2007	43		Exotoxin A		19
			Alkaline Protease		30
			Elastase		14
			Combination of 3 antigens		42

Antibody response in intermittent Pseudomonas aeruginosa infection

From longitudinal studies, it is known that elevated antibody titres are not only observed in chronic infections, but also in an early stage of infection. Intermittent colonisation may give rise to formation of specific antibodies. However, lower titres are found in patients with intermittent colonisation compared to chronic colonisation.[50;52] As antibody titres are also influenced by antimicrobial treatment, titres may vary. Increasing titres are a sign of development of chronic infection.[58;59]

Antibody response in early Pseudomonas aeruginosa infection

Table 5 describes the numbers of patients that have evidence of anti-*P. aeruginosa* antibodies before a first positive culture. The early antibody response is strongly dependent on the method used. In a study performed by Burns et al, the time of first detection of antibody for both exotoxin A and whole cell protein immunoblot preceded the detection of *P. aeruginosa* by culture in some patients. By 3 years of age, 97,5 % of patients showed evidence of *P. aeruginosa* infection, measured by a combination of two serological assays, BALF- and oropharyngeal culture. As determined by culture only, 72,5 % of patients demonstrated evidence of *P. aeruginosa* during their first three years.[11] Increase of anti-*Pseudomonas* antibodies occurred even 6-12 months before *P. aeruginosa* was isolated from cultures of the respiratory tract in young children in another study. Development of antibodies before isolation of *P. aeruginosa* occurred in approximately 30-70 % of patients.[16;42;55;56]

Table 5 Percentage of patients with raised antibody titres before the isolation of *P. aeruginosa* from respiratory cultures

Author	Sample size	Test	Antigen	Percentage
Brett '88	33	ELISA	Whole cell protein	73
West '02	52	ELISA	Exotoxin A	54
			Cell lysate	63
Kappler '06	3	ELISA	Combination of 3 antigens	33
Tramper-Stranders '06	13	ELISA	Combination of 3 antigens	38

Conclusion

Early detection of *P. aeruginosa* in patients with CF is of major importance because aggressive antimicrobial therapy might prevent or postpone chronic *P. aeruginosa* infection and subsequent disease progression. Qualitative culturing has limitations, especially regarding site of culturing and sampling technique. Predictive values of oropharyngeal cultures can be improved by culturing more regularly. Sputum induction in children has little additional value for detection of *P. aeruginosa* in comparison with conventional culture of sputum or oropharyngeal swabs. Advantages of molecular techniques are a high diagnostic values and the possibility of early detection of respiratory pathogens. Serological tests are use-

ful parameters for monitoring of *P. aeruginosa* infected patients as titres may vary with antimicrobial treatment and progression of disease. Antibodies against *P. aeruginosa* may emerge months before a culture becomes positive, but this is not a rule. A combination of serological testing with molecular methods could be helpful, particularly for symptomatic patients without positive cultures.

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Boy at Jal Mahal - Jaipur, India

5

Diagnostic value of serological tests against *Pseudomonas aeruginosa* in a large cystic fibrosis population

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Abstract

Serological methods to monitor *Pseudomonas aeruginosa* colonisation in patients with cystic fibrosis are advocated but the diagnostic value of a commercially available *P. aeruginosa* antibody test to detect early and chronic *P. aeruginosa* colonisation in a non-research setting has not been assessed.

Colonisation with *P. aeruginosa* was estimated by regular culture of sputum or oropharyngeal swabs during three consecutive years in 220 patients with CF aged 0-65 years. Commercially available ELISA tests with three *P. aeruginosa* antigens (elastase, exotoxin A, alkaline protease) were performed at the end of the study period. In a subgroup of 57 patients (aged 4-14 years) serological tests were performed annually.

Using culture as the reference standard, the ELISA tests using the advised cut off values had a sensitivity of 79 % and a specificity of 89 % for chronic colonisation. Receiver-operator characteristic curves were created to optimize cut off values. Applying these new cut off values resulted in a sensitivity of 96 % and a specificity of 79 %. All three individual serological tests discriminated well between absence and presence of chronic *P. aeruginosa* colonisation. The sensitivity of the individual antibody test was 87 % for elastase, 79 % for exotoxin A and 76 % for alkaline protease. First colonisation was preceded by positive serological results in only five of thirteen patients (38 %).

In patients with CF, serological tests using specific antigens are sensitive for diagnosing chronic *P. aeruginosa* colonisation. However, the failure of serological tests to detect early colonisation in young patients emphasises the need for continued reliance on cultures.

Introduction

Pulmonary colonisation with biofilm-producing strains of *P. aeruginosa* in patients with cystic fibrosis (CF) is associated with a decline in pulmonary function and subsequent morbidity and mortality.[1;2] In infancy, 10-30 % of patients are colonised with *P. aeruginosa*, and this increases to 80-90 % during adolescence.[3;4] Initial colonisation and early asymptomatic infection are close entities; these precede chronic colonisation and infection. The transition duration of initial asymptomatic colonisation and infection to chronic tissue-destroying infection and colonisation varies among patients. Therefore in this paper, the term 'colonisation' is used. It is of major importance to detect *P. aeruginosa* at an early stage since aggressive treatment of early colonisation might delay or prevent chronic colonisation.[5;6] Acquisition of *P. aeruginosa* is often monitored by culture of sputum or oropharyngeal swabs. Serological methods such as Crossed Immune Electrophoresis (CIE), Western immunoblot and Enzyme Linked Immuno Assay (ELISA) to detect *P. aeruginosa* are not routinely used in CF centres.[7-9] Precipitin measurement by CIE has been largely taken over by ELISA and Western blotting techniques. CIE and whole cell protein ELISA have a high sensitivity (96-100 %) for chronic colonisation.[10] ELISA with purified antigens has a lower sensitivity (15-100 %), depending on the antigen and stage of colonisation.[11] Recently prospective studies suggested that antibodies may be present before the first positive culture.[12-14]

Antibody development is influenced by the immunological condition of the host, corticosteroid use, antibiotic treatment targeted against *P. aeruginosa* and *P. aeruginosa* related factors such as phenotype and production of exoproteins.[15-17] Only a few data are available on the clinical relevance of anti-*P. aeruginosa* antibody responses. Obviously, these antibodies fail to eliminate *P. aeruginosa* and lack protective effects.[18;19] The antibody response seems to be more prominent in patients with severe clinical disease, suggesting they might play a pro-inflammatory role.[20]

In this study, we assessed the value of a commercially available serological test using 3 purified antigens to detect chronic *P. aeruginosa* colonisation in a large CF population. We also examined the value of these serological tests for early detection of *P. aeruginosa* colonisation.

Methods

Participants

We performed the study in the CF Centre Utrecht, the Netherlands. All patients with a diagnosis of CF, confirmed by sweat chloride test > 60 mmol/L and/or genotyping were eligible to participate in the study. Fifty two adults and 168 children (age < 18 years) participated in the cross-sectional study; 57 of the 168 children also participated in a 3-year prospective study.

The medical ethics committee of the University Medical Centre Utrecht approved the study. All participants or their parents gave written informed consent.

Cultures

Sputum or oropharyngeal cultures were carried out for all patients according to the treatment protocol of the centre in three consecutive years from January 2002. When a patient was not able to produce sputum, an oropharyngeal swab was taken. The sensitivity and positive predictive value of oropharyngeal swabs ranges from 44 % to 83 %. However, for the classification of colonisation status, negative predictive values (percentage of patients with a negative test (oropharyngeal culture) who do not have the disease (*P. aeruginosa* colonisation)) are more important. These negative predictive values range between 85 % and 97 %.[21-23] The mean number of cultures per patient was six (median 4, range 3-43), depending on pulmonary condition.

Culture samples were inoculated onto blood- and McConkey agar plates. After incubation at 37°C, media were inspected for any growth of *P. aeruginosa* after 24 and 48 hours. Classification of *P. aeruginosa* colonisation status of patients over the 3 year period was based on the criteria of Lee et al[24] (chronic colonisation: > 50 % of all cultures positive; intermittent colonisation: ≤ 50 % of all cultures positive, with at least one positive culture; no colonisation: all cultures negative for *P. aeruginosa*).

ELISA

At the end of the observation period in 2004, serum samples were obtained from the cross-sectional study group. In the prospective cohort of 57 paediatric patients, annual serum samples were drawn concomitantly when cultures were taken. All sera were stored at -20°C.

Serological testing was performed using a commercially available ELISA-kit (Mediagnost, Reutlingen, Germany). This semi-quantitative IgG ELISA consists of three common *P. aeruginosa* antigens: elastase, exotoxin A and alkaline protease. In brief, serum samples were diluted by a factor 103 with phosphate buffered saline (PBS). In each well of a 96-well microtitre plate, 100 µl of a diluted sample was added and incubated at 37°C for two hours. After aspirating and washing three times intensively with PBS/Tween-20, 100 µl of conjugate solution (anti-human IgG peroxidase) was added to each well. The plate was further incubated for 2 hours at 37°C. After washing thoroughly, wells were filled with 100 µl substrate solution (tetramethyl benzidine) and again incubated at room temperature in the dark. After half an hour the reaction was stopped by sulphuric acid. Within ten minutes, the optical density was read by a photometer set at 450 nm. Titres were extrapolated from optical density values of two negative and two positive control serum samples according to the manufacturer's manual.

Lung function

Clinical data for every CF patient are collected in a database annually. Data collected at the end of the observation period in 2004 were used to compare lung function of patients chronically colonised with *P. aeruginosa* with non-colonised patients (forced expiratory volume in 1 second as percentage of predicted (FEV_1 % pred) and forced vital capacity as percentage of predicted (FVC % pred)).

Statistics

The ELISA manual advised a cut off titre against one or more of the tested antigens of 1:500 or 1:1250 (borderline positive or positive, respectively). When applying these cut off titres to our study group, low sensitivities of the ELISA for chronic *P. aeruginosa* colonisation, with cultures as a reference standard, were found. The ability of the serological tests to discriminate between chronic colonisation and no colonisation was therefore estimated using the area under the receiver operating characteristic curve (ROC area). Difference in discriminatory power between the individual serological tests was estimated by calculating the difference in ROC area (ΔROC area) with 95 % confidence intervals, taking into account the correlation between the expressions as they were based on the same cases. Cut off titres with best discrimination (defined as the point on the ROC curve closest to the upper left corner) were used to classify patients as having negative or positive serological test results.[25] The lung function data of chronically colonised and non-colonised patients were compared using the Student's *t* test, and multiple linear regression. These parameters were expressed as mean (SD). Differences were considered significant if the p value exceeded 0.05. Calculations were performed by Statistical Package for the Social Sciences (SPSS) version 12.0 (Chicago, IL, USA).

Results

Participant characteristics

The mean (SD) age of the patients in the cross-sectional study at the end of the observation period was 14.5 (10.6) years, and for those in the prospective study was 8.1 (2.8) years.

Table 1 Characteristics of participants in the cross-sectional and prospective studies

Cross-sectional study		
Number of participants		220
Mean (SD) age (years)		14.5 (10.6)
Median (range) age (years)		11.8 (0.7-65.4)
Age < 18 years, n (%)		168 (76)
Male, n (%)		110 (50)
P. aeruginosa colonisation status, n (%)	Chronic	67 (31)
	Intermittent	60 (27)
	No	93 (42)
Prospective study		
Number of participants		57
Mean (SD) age (years)		8.1 (2.8)
Median (range) age (years)		7.9 (4.3-14.2)
Male, n (%)		30 (53)
P. aeruginosa-colonisation status, n (%)	Chronic	13 (23)
	Intermittent	13 (23)
	No	31 (54)

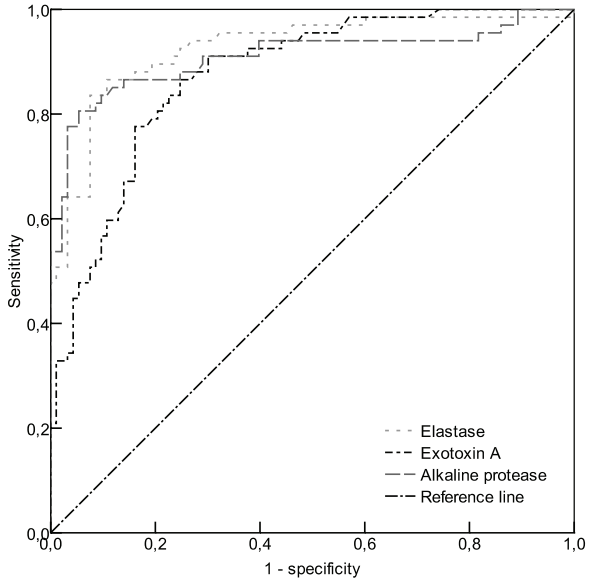
Sixty-seven patients had chronic colonisation with *P. aeruginosa*, sixty were intermittently colonised and ninety-three patients were not colonised. The characteristics of the study population are summarized in table 1. The impact of chronic *P. aeruginosa* colonisation on clinical characteristics was estimated. Mean FEV_1 % pred and FVC % pred were significantly lower in chronically *P. aeruginosa* colonised patients than in non-colonised patients (FEV_1 % pred 64.8 (29.2) *v.* 89.0 (19.4); FVC % pred 80.0 (22.4) *v.* 94.1(13.4); $p < 0.001$, adjusted for age).

Diagnostic value of serological tests for chronic P. aeruginosa colonisation

The ELISA manual regarded a titre of > 1:500 against one or more of the tested antigens as borderline positive and a titre of > 1:1250 as positive. With these cut off titres, sensitivity for chronic colonisation was 79 % and 66 %, respectively. The specificity was 89 % and 96 %, respectively.

The ROC curves (figure 1) show that all three serological tests discriminate well between colonised and non-colonised patients. The ROC area was highest for elastase (0.926), followed by alkaline protease (0.909) and exotoxin A (0.874). There were no significant differences in discriminatory power (ROC areas) between the three serological tests.

Figure 1 Receiver-operator characteristic (ROC) curves of three individual serological tests



Test characteristics associated with best discriminatory cut off titres are shown in table 2. Although alkaline protease has a higher ROC area than exotoxin A, it has a somewhat lower sensitivity (79 % *v.* 76 %).

Table 2 ROC areas and cut off titres with test characteristics

	Area	95 % CI	Best cut off titre*	Sensitivity	Specificity
Elastase	0.926	0.882-0.969	> 1:35	87 %	89 %
Exotoxin A	0.874	0.821-0.927	> 0	79 %	81 %
Alkaline protease	0.909	0.856-0.963	> 0	76 %	97 %

** point closest to the upper left corner on the ROC-curve*

Positive serological results for elastase, exotoxin A and alkaline protease were found in 87 %, 79 % and 76 %, respectively, of patients with chronic colonisation compared with 42 %, 48 % and 17 % of patients, respectively in the intermittently colonised group and 11 %, 19 % and 3 %, respectively in the non-colonised group. Combining results of individual serological tests, 96 % of chronically colonised patients had at least one positive antibody titre. Elastase serology was the most sensitive, but six elastase antibody negative patients appeared positive for other *P. aeruginosa* antibodies if a combination of antibody tests was used. The specificity of the combination of serological tests was 79 %. Test characteristics calculated with the cut off values for borderline positivity of the ELISA manual and test characteristics using the cut off values listed in table 2 were compared: the sensitivity increased from 79 % to 96 %, while the specificity decreased from 89 % to 79 %.

Prevalence of P. aeruginosa positive cultures and positive serological tests

To measure the additional value of serological tests in comparison with cultures to detect *P. aeruginosa*, data of cultures and serological tests from different age cohorts were analysed. One or more *P. aeruginosa* positive cultures and/or positive serological tests were observed during the 3-year study period in 20 % of the youngest patients (aged 0 to 2 years), and in 81 % of the adult group (> 18 years) (figure 2a). All the paediatric age groups had more patients with positive cultures than with positive serological tests. In the two youngest age groups (0 to 2 and 3 to 5 years), no additional *P. aeruginosa* was detected by serology. From age cohort 6 to 8 years and older, evidence for additional *P. aeruginosa* colonisation was obtained by serological testing rather than from culture alone in 3 to 11 %. Chronic colonisation for each age group is shown in figure 2b.

Figure 2a Prevalence of *P. aeruginosa* positive cultures and positive serological results per age cohort

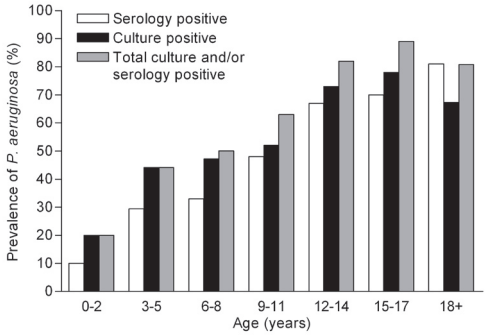
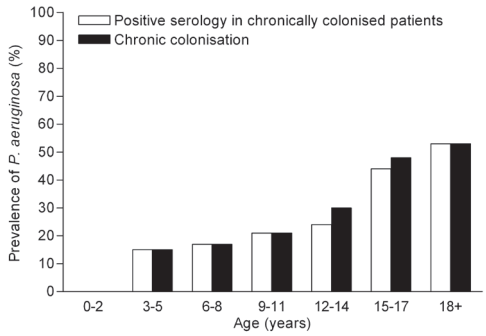


Figure 2b Prevalence of chronic *P. aeruginosa* colonisation per age cohort and positive serological results in chronically colonised patients



None of the youngest patients (0 to 2 year) and 53 % of the adult cohort (> 18 years) were chronically colonised with *P. aeruginosa*. Positive serological results accompanied chronic colonisation in all age cohorts except for the 12 to 14 and 15 to 17 year cohort.

Value of serological tests in early detection of P. aeruginosa colonisation

Prospective data of 57 children show that 15 of these patients had a culture conversion during the follow-up period. Data of these patients are shown in table 3. In this group, 5 patients showed positive serology prior to a positive culture, 7 patients remained seronegative and 1 patient had a simultaneous conversion for both culture and serology. Timing of positive serology did not depend on age. No uniform antibody pattern was seen in early detection by positive serology (table 3). Evidence for positive serology at first positive culture and height of antibody titres could not predict *P. aeruginosa* colonisation to become transient or chronic. Ten patients from the prospective cohort showed a transient positive serology without any following positive culture.

Table 3 Serological patterns in patients with culture conversion during follow-up period

Patient number	2001	2002			2003				2004				2005	
	Pa*	E†	EA	AP	Pa	E	EA	AP	Pa	E	EA	AP	Pa	Pa
1	0	-	+	-	0	-	-	-	1	-	-	-	1	0
2	0	-	+	-	0	+	+	-	0	-	+	-	1	0
3	0	+	-	+	0	+	-	+	1				1	
4	0	-	-	+	0	-	-	-	0	+	+	+	1	1
5	0	+	-	-	0	-	-	-	1	+	+	+	1	1
6	0	-	-	-	0	-	-	-	0	-	-	-	1	1
7	0	-	-	-	0	-	-	-	1	-	-	-	0	0
8	0	-	-	-	0	-	-	-	1	-	-	-	0	0
9	0	-	-	-	0	-	-	-	1	-	-	-	0	0
10	0	-	-	-	0	-	+	-	1	+	+	-	1	1
11	0	-	-	-	1	-	-	-	0	-	-	-	0	0
12	0	-	-	-	1	-	-	-	0	-	-	-	0	1
13	0	-	-	-	0	-	-	-	1	-	-	-	0	0
14	0	+	-	-	1	-	-	-	1				0	1
15	0	+	+	+	1	+	+	-	0	+	+	-	0	0

**Pa=P. aeruginosa in culture (0=no, 1=yes)*
†E=elastase; EA=exotoxin A; AP=alkaline protease; + = positive serology; - = negative serology

Discussion

To our knowledge, this is the first study to address the value of a commercially available ELISA test for detecting *P. aeruginosa* colonisation in a large CF population. Using cut off values according to the manufacturer's manual, this serological test has relatively low sensitivity for the detection of chronic *P. aeruginosa* colonisation. After adjusting cut off values, extrapolated from ROC curves from our own population, serological tests against common *P. aeruginosa* antigens had a higher sensitivity without significant loss in specificity. However, from the prospective cohort data it seems that the additional value of serological testing for diagnosis of early *P. aeruginosa* colonisation in comparison with successive cultures is limited, especially in young children.

Colonisation with *P. aeruginosa* is a clinically relevant issue; *P. aeruginosa* colonisation has a significant impact on lung function, even when adjusted for age. These data confirm previous studies that show worsening of clinical parameters after acquisition of *P. aeruginosa*.[26]

To detect *P. aeruginosa* colonisation, measurement of *P. aeruginosa* antibodies is advocated. Previous studies show high sensitivity and specificity of serological tests for chronic *P. aeruginosa* colonisation, depending on the serological method used.[27-29] However, some of these studies included very small numbers of patients and test characteristics can be influenced by different definitions of *P. aeruginosa* colonisation. Culture is often used as a reference standard. Culture sampling errors may occur and therefore may influence the test characteristics of the serological tests. We therefore examined cultures during three consecutive years to make a clear division between non-colonised and chronically colonised patients.

The ROC curves show that elastase, exotoxin A and alkaline protease serological tests all discriminate well between colonisation and non-colonisation. Test characteristics depend on cut off values. Instead of using the cut off values suggested by the ELISA manual, new cut off values were estimated from the ROC curve to obtain the best discrimination. Using these cut off values, we calculated a higher sensitivity in comparison with the use of cut off values advised by the manufacturer. With regard to the clinical relevance of *P. aeruginosa* colonisation, the sensitivity of the test need to be high. When a positive serological result appears without other signs of *P. aeruginosa* infection, close monitoring of cultures can be performed to detect any underlying *P. aeruginosa* colonisation.

Elastase serology is the most sensitive test for chronic colonisation, with a sensitivity of 87 %. Hollsing *et al*, using a different ELISA assay, found a sensitivity of elastase of only 23 % for chronic colonisation in a small study cohort.[30] A possible explanation for the large difference is the definition of chronic colonisation used. They used a classification of colonisation status based on cultures over a 6-month period prior to serological measurement while we defined colonisation over a 3 year period. Another explanation might be the low cut off titres that we used but, even with the cut off titres advised by the manufacturer's manual, the sensitivity of our test was much higher. The sensitivity of the other two antibodies is also higher in our study than in other studies. Exotoxin A had a sensitivity of 79 %, but the specificity was lowest of the three antigens. Alkaline protease had the lowest sensitivity (76 %) but a very high specificity (97 %) and might therefore be useful to rule out *P. aeruginosa* colonisation.

Single antigen ELISAs directed against *P. aeruginosa* virulence factors fail to show an immu-

nological response in all *P. aeruginosa* colonised patients. In our study the detection rate increased from 87 % to 96 % when compared with single antibody testing with elastase.
With regard to the use of successive cultures as reference standard, problems with definition of specificity may arise because early *P. aeruginosa* colonisation might not yet be proved by positive cultures although the serological test is positive. On the other hand, a transient positive serological result without positive cultures was also seen in our follow-up cohort. We did not determine antibodies in healthy controls, but as *P. aeruginosa* is a common pathogen from the environment, it is possible that contact with these bacteria leads to transient antibody formation. Healthy controls might have positive antibody titres, as was shown by Pedersen et al.[31]
In children aged 4 to 6 years, serological tests were of no additional value for diagnosing *P. aeruginosa* colonisation. Older patients have a higher prevalence of antibodies in the absence of positive cultures. At ages 6 to 17 years, only a small proportion of patients (3 to 11 %) had positive serological results without a positive culture. The prevalence of *P. aeruginosa* colonisation in the youngest patients was lower than described in recent literature. Burns *et al* showed that 72.5 % of patients had evidence for *P. aeruginosa* colonisation by culture and 97.5 % by both culture and serology before the age of 3 years. In the study of Li *et al*, almost 90 % of 3-year old patients had acquired *P. aeruginosa* in their cultures.[32;33]
The prospective data of 57 children show that, in case of culture conversions to a positive culture, no uniform serological pattern is seen. From the 13 patients who showed culture conversion, only five showed early positive serological tests that could have been helpful in diagnosis. Thus, serological methods with one or more individual antigens as a screening tool for early *P. aeruginosa* colonisation in a clinical setting do not seem to be very sensitive. The failure of serological tests to detect early colonisation in young patients emphasises the need for continued reliance on culture. West *et al* also found that only 54 % of children had positive exotoxin A serology before or at the time a culture became positive.[34]
In our cross sectional study the antibody that was raised most often in non-colonised patients was exotoxin A, but in the longitudinal follow up, it was not clear that exotoxin A titres increased before other titres. The height of antibody titres could not predict whether early colonisation would become chronic. The cohort may have been too small to show a relation between increasing antibody titres and chronic *P. aeruginosa* colonisation.
In the majority of patients with positive serological tests and negative culture the antibodies disappeared. It seems that single positive antibody titres without concomitant positive cultures do not predict whether a patient will become colonised.

In conclusion, serological tests using specific antigens are sensitive for diagnosing chronic *P. aeruginosa* colonisation in patients with CF but, except in study settings, they are not of great additional diagnostic value in comparison with successive cultures. The antibody elastase is most sensitive for chronic colonisation. In early colonisation no uniform serological pattern is seen; titres might be raised before a culture becomes positive, but this is not a rule. To detect early colonisation in young CF patients, we would favour regular culturing instead of serological testing with specific antigens.

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Children selling fish - Lake Nyasa, Tanzania

A controlled trial of cycled antibiotic prophylaxis to prevent initial *Pseudomonas aeruginosa* infection in children with cystic fibrosis

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Submitted

Abstract

Initial pulmonary *Pseudomonas aeruginosa* infection in cystic fibrosis (CF) patients is currently treated with intensive antibiotic therapy. At this stage inflammation and tissue injury might have already occurred. Moreover, bacterial eradication is not always achieved. Prophylactic treatment against *P. aeruginosa* seemed to have a preventive effect in retrospective studies. Our aim was to prospectively establish the effect of cycled prophylactic treatment on prevention of initial *P. aeruginosa* infection in paediatric CF patients.

This 3-year triple-blinded randomised controlled trial included 65 children with CF without *P. aeruginosa* infection. Intervention existed of 3-monthly 3-week treatments with oral ciprofloxacin and inhaled colistin or both placebo controls. Primary outcome was *P. aeruginosa* infection. Secondary outcomes were serum anti-*Pseudomonas* antibodies, pulmonary function, exacerbations, chest-X-ray scores, inflammation parameters, respiratory pathogens and antimicrobial resistance.

There was no difference in acquisition of *P. aeruginosa* infection (32 % control group versus 26 % treatment group; hazard ratio 0.738; 95 % CI 0.299-1.822). Anti-*Pseudomonas* antibodies emerged earlier in the control group; after 3 years this difference had disappeared. After 3 years, chronic infection was observed in 19 % of control- and 12 % of treated patients. Pulmonary function decline and other clinical outcomes were not different between the two groups. In the treatment group significantly less Gram-positive bacteria and *Enterobacteriaceae* but more non-*P. aeruginosa* non-fermentative Gram-negative bacteria were observed.

Three-monthly cycled anti-*P. aeruginosa* prophylaxis does not reduce the risk of initial and chronic infection in *P. aeruginosa*-negative children with CF of all ages. Shifts in bacterial colonisation demand caution.

Introduction

Children with cystic fibrosis (CF) are at high risk to acquire pulmonary infections with *Pseudomonas aeruginosa*. A combination of host susceptibility and bacterial adaptation make *P. aeruginosa* hard to eradicate once established in the CF airways.[1-3] Infection with *P. aeruginosa* is associated with irreversible pulmonary damage which is elicited by a primarily neutrophil-dominated inflammatory response and is a major predictor for mortality and morbidity.[4-6]

P. aeruginosa infection and pulmonary inflammation often precede respiratory symptoms; inflammation is more pronounced in infections with *P. aeruginosa* than with other organisms.[7-10] Children are already infected at a young age; figures range from 28 to 85 % at 3 years of age depending on isolation methods.[4;7;9] A relation between *P. aeruginosa* infection and diminished lung function was observed in young children even when *P. aeruginosa* was apparently eradicated after initial infection.[11]

Currently, initial detection of *P. aeruginosa* in children with CF is managed with intensive antimicrobial therapy. Eradication of the pathogen from CF airways might be achieved when antibiotic treatment is initiated early after pulmonary acquisition.[9;12-19] The optimal treatment regimen has not yet been established; there is a lack of randomised controlled trials comparing several regimens of antimicrobial therapy.[19;20] Despite early antibiotic therapy, eradication fails in 10 to 40 % of cases and these patients are predisposed for chronic infection with *P. aeruginosa*. Additionally, when eradication is successful, re-infection often occurs within one year, posing the patient at risk for further deterioration of pulmonary function. Limited evidence from two retrospective studies indicated that continuous prophylactic antibiotic therapy against *P. aeruginosa* diminished the number of patients with initial and chronic *P. aeruginosa* infections.[21;22]

The facts that *P. aeruginosa* infection also in early life is associated with irreversible pulmonary damage and that early eradication therapy might fail, prompted us to prospectively evaluate the effectiveness of prophylactic antibiotic therapy to prevent initial *P. aeruginosa* infection and subsequent pulmonary function decline. We hypothesised that cycled prophylactic anti-*Pseudomonas* treatment of *P. aeruginosa*-negative CF patients would either prevent or delay the first acquisition of *P. aeruginosa* or eradicate the organism in a very early phase before the onset of chronic pulmonary infection, accompanying pulmonary inflammatory response and bacterial adaptation.

Methods

Patients and settings

Children between 0 and 18 years of age were recruited from four tertiary CF centres in the Netherlands. Eligibility criteria were CF as confirmed by positive sweat chloride test and/or genotyping; *P. aeruginosa* negative state, determined as absence of *P. aeruginosa* positive cultures and/or serology and absence of regular treatment with anti-*Pseudomonas* antibiotics in the previous 2 years. The institutional review board at each centre approved the study.

Parents and children above the age of 12 years provided written informed consent before study participation.

Study design and randomisation

We conducted an investigator-initiated parallel group randomised placebo-controlled trial with triple blinding (investigators, physicians and participants). Participants were randomly assigned by a computerised program in blocks of four, stratified according to centre and age (0 to 5; 6 to 11; 12 to 18 years). Randomisation and packaging of study interventions was done by the independent pharmacy department of the University Medical Centre Utrecht. The randomisation code was only disclosed after completion of all follow-up data and data entry in a study database.

Study intervention

The study duration was three years. The intervention existed of colistimethate sodium inhalation 80 mg *bid* combined with oral ciprofloxacin 10 mg/kg *bid* or both placebo controls for continuous periods of three weeks every three months. Within a three-year study period, there were twelve study intervention cycles. We choose this eradication regimen because of extensive experience as eradication therapy, also among children, in CF centres across Europe. Colistimethate sodium powder 80 mg (1 million units) per dose, and an identical placebo containing 80 mg mannitol powder both with 3 ml normal saline as solvent were provided by Grünenthal, Aachen, Germany. Colistimethate sodium or placebo was inhaled by a perforated vibrating membrane nebulizer (eFlow Rapid, PARI, Starnberg, Germany) and if necessary preceded by salbutamol inhalation.[23] Ciprofloxacin granules and corresponding identical placebo (coated sugar-sphere granules) with sweet-flavoured suspension were provided by Bayer Schering Pharma, Berlin, Germany. Both pharmaceutical companies only provided study medication and did not participate in study design, conduct, analysis, writing and review of the manuscript.

Follow-up during the study

Follow-up visits were performed 3-monthly, between two and five weeks after the study intervention. At each visit, a respiratory specimen (oropharyngeal cough swab or sputum) was collected for microbial culture. Number of pulmonary exacerbations, antimicrobial agent use, height, weight and adverse events were noted. Additionally, pharmacists were addressed to provide an overview of antimicrobial agent use during the study period to prevent recall bias. Spirometric tests for children aged 4 years and older were performed 6-monthly. Pulmonary function was assessed as forced expiratory volume in 1 second as percentage predicted for height and sex (FEV_1 %) and forced vital capacity as percentage predicted for height and sex (FVC %) after 800 µg salbutamol inhalation, using Zapletal reference data.[24] Serum samples were collected every 6 months for measuring serum anti-*Pseudomonas* antibodies. Chest radiography, complete blood count, C-reactive protein and serum immunoglobulin measurements were performed annually. Other standard CF care was maintained throughout the trial.

Microbiology

Respiratory specimens were cultured onto appropriate media. *Pseudomonas* species were identified by a positive oxidase reaction. Further identification of *P. aeruginosa* was performed by measuring C-390 resistance.[25] Susceptibility testing was performed by disk diffusion using Neo-Sensitabs (Rosco, Taastrup, Denmark) and was interpreted according to CLSI breakpoints.

Serological testing was performed by ELISA with 3 common *P. aeruginosa* antigens: elastase, exotoxin A and alkaline protease (Mediagnost, Reutlingen, Germany) as described before.[26] A titre of > 1:1250 for one or more of the antigens was considered positive, as described in the ELISA manual.

Outcomes

The primary efficacy outcome was *P. aeruginosa* infection, as determined by two positive cultures taken > 1 week apart (to increase positive predictive value for pulmonary *P. aeruginosa* infection in case of oropharyngeal cough swabs) or, at the discretion of the treating physician, one positive culture and a severe pulmonary exacerbation that required rapid antimicrobial treatment. When the primary endpoint was reached, the participants were abstained from further study intervention and received standard anti-*Pseudomonas* treatment. All participants that reached the primary endpoint or that withdrew from study interventions were included in the follow-up. Serological test results were not included in the primary efficacy endpoint.

Secondary efficacy outcomes included anti-*Pseudomonas* antibodies; pulmonary function; body mass index; number of pulmonary exacerbations (defined by increased cough and/or sputum production and/or decreased weight and/or changes in chest auscultation requiring antimicrobial therapy); days of antibiotic use (all antibiotic treatments; anti-*Pseudomonas* antibiotics were only prescribed in case of reaching the primary efficacy outcome); adverse events; antimicrobial resistance; bacterial colonisation, chest X-ray (adjusted Crispin Norman[27]) scores and inflammation parameters.

Statistical analysis and power calculation

We estimated that with 32 children in each group, the study would have 80 % power to detect 75 % reduction in *P. aeruginosa* infections in the treatment group at a 2-sided alpha level of 0.05; assuming a *P. aeruginosa* acquisition rate of 15 % per year in the control group.

The trial was analysed by the principal investigator according to the intention to treat principle. In case of participants reaching the primary efficacy endpoint, data with respect to cultured pathogens and antimicrobial agent use were analysed from the start of the study till the first full study year after having reached the endpoint. Survival analysis was executed with construction of Kaplan-Meier curves and Cox proportional hazard model regression with and without time-dependent factors and age and baseline FEV_1 % as stratifying variables. Nominal data were analysed using chi-square or Fisher's exact test; continuous data with Students *t* test or Mann Whitney U test. Data are shown as percentages or mean ± standard error of the mean.

Longitudinal data with repeated measurements (pulmonary function and antibody titres) were analysed with a mixed effects model with random participant-specific slope and intercept. Baseline FEV_1 % and age were included as covariates in the pulmonary function model. An interaction term between allocation and time was examined in order to determine whether decline in pulmonary function or increase in antibody titres differed between allocation groups.
Statistical analysis was performed with SPSS version 15.0 (Illinois, Chicago, USA).

Results

Study group

Sixty-five patients were randomised and entered the study. Figure 1 shows the flow diagram of inclusion. Baseline characteristics for both the control- and treatment group were similar for the total group as well as for subgroups of different ages except for FEV_1 % (p=0.01; table 1).

Figure 1 Flow diagram explaining the recruitment and selection of participants

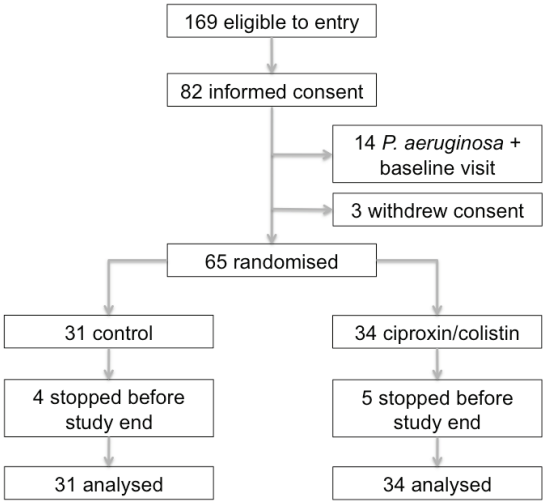


Table 1 Baseline characteristics of participating children with CF without *P. aeruginosa* infection

	Control	Treatment
Number	31	34
Age	7.79 ± 0.68	6.69 ± 0.67
Female sex	16 (52)	14 (41.2)
CF-genotype		
dF508/dF508	19	22
dF508/other	11	10
other/other	1	2
Sputum producer	12 (38.7)	5 (14.7)
S. aureus colonisation	15 (48)	13 (38)
Anti-staphylococcal prophylaxis	21 (68)	18 (53)
Ever *P. aeruginosa* in culture > 2 years before study entry	6 (19.4)	7 (20.6)
FEV_1 % ± SE	94 ± 3.0	107 ± 3.7
BMI z-score ± SE	-0.16 ± 0.17	0.25 ± 0.14

Data indicated as mean (SD) or N (%).

P. aeruginosa infection

Nineteen of 65 patients (29 %) reached the primary endpoint during the study period; 10/31 (32 %) in the control group and 9/34 (26 %) in the treatment group. The hazard ratio for the treatment group to acquire *P. aeruginosa* infection was 0.738 (95 % CI 0.299 to 1.822); p=0.510. Mean duration to *P. aeruginosa* infection was 0.86 ± 0.21 year for the control group and 1.29 ± 0.19 year for the treatment group (p=0.158). Figure 2a shows the survival curve. The hazard ratios were not different between the groups over time (p=0.101). The median age of acquiring first *P. aeruginosa* infection was 6.8 year (range 2.45 to 18.42 years). Patients with previous *P. aeruginosa* isolation more than two years before study entry did not more often reach the primary endpoint (p=0.912).

Of the nineteen participants that reached the primary efficacy endpoint, four participants had one *P. aeruginosa* positive swab culture accompanied by an exacerbation. Three of the four patients with an exacerbation had a second positive culture subsequently. In four of the nineteen patients, *P. aeruginosa* was detected in sputum (3 control group, 1 treatment group). Three other participants had once *P. aeruginosa* in a swab culture without an exacerbation, follow-up cultures remained negative and therefore the primary endpoint was not met.

Eradication of *P. aeruginosa* in patients that had reached the primary endpoint and ceased the study interventions to obtain usual anti-*Pseudomonas* treatment was established in 60 % of control group patients and 67 % of treatment group patients after one or more eradication cycles (p=0.764).

Figure 2a Kaplan-Meier survival curves of the primary efficacy endpoint (N=65)

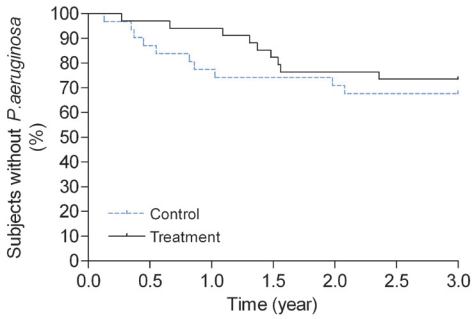
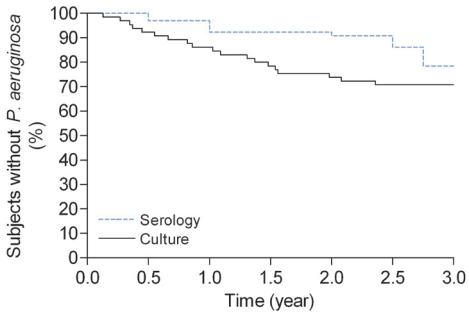


Figure 2b Kaplan-Meier survival curves for the entire study group (N=65) with respect to timing of culture positivity and serology positivity



P. aeruginosa serology

The proportion of patients having a positive antibody titre was higher in the control group during the first year (p=0.021), in the second and third year the differences between the two groups disappeared. As calculated by a mixed model, the sum of three serum anti-*Pseudomonas* antibodies was not different (p=0.08). In general, positive antibody titres followed positive cultures and were strongly associated with a positive culture in the past (figure 2b). In total, fourteen children had once or more often positive antibody titres throughout the study period. Exotoxin A antibody titres were elevated in ten patients, followed by alkaline protease (five patients) and elastase (three patients). Ten of the fourteen children with positive antibody titres were culture positive before serology positive. Two patients showed intermittent positive antibody titres without any positive cultures during the study period and two other patients showed positive antibody titres without positive cultures till date (both two in control- and treatment group).

Chronic infection, estimated as > 50 % of evaluable months *P. aeruginosa* in cultures for at least one year and elevated antibody titres in the last study year, was observed in 19 % of patients from the control group and 12 % of patients from the treatment group.

Secondary clinical outcomes

If corrected for baseline FEV_1 % and age, the average FEV_1 % was not higher in the treatment group during the study (p=0.206). The rate of decline in pulmonary function (FEV_1 %) over the 3-year study period in the treatment group was not significantly different from the control group (p=0.94), being -8.3 % for the control group and -10.4 % for the treatment group. Older children did not have a faster decline in FEV_1 % than younger children. In the first study year, 67.7 % of the control group and 58.8 % of the treatment group required one or more antibiotic treatments because of a pulmonary exacerbation or reaching the primary endpoint. The median time to first exacerbation requiring antibiotic therapy was 0.75 year for the control group versus 1.00 year for the treatment group (p=0.223). The number of intravenous antibiotic treatments during the 3-year study period did not differ between groups (17 % in the control group and 14 % in the treatment group). The mean number of days on antimicrobial therapy because of respiratory exacerbations (excluding maintenance antibiotics) for the total study period was 66 ± 9.52 for the control group and 67 ± 9.92 for the treatment group. The figures of the other secondary outcomes can be found in table 2.

Table 2 Outcome of clinical parameters

	Control	Treatment	p-value
FEV_1 % decline during study period	-8.3	-10.4	0.940
BMI z-score change	-0.09 ± 0.15	-0.003 ± 0.08	0.603
Chest X-ray score change	+2.10 ± 0.51	+2.00 ± 0.50	0.885
Inflammatory parameters			
Immunoglobulin G	9.84 ± 3.54	8.66 ± 3.82	0.105
Leucocytes (x 10^9/l)	9.17 ± 2.88	8.67 ± 2.68	0.499
Proportion of patients with CRP > 10 mg/l during study	16.1	14.7	0.874
Adverse events	45.2	50.0	0.696
Cough and/or increased sputum production	38.7	23.5	0.185
Gastro-intestinal	9.7	20.6	0.192
Other (headache. erythema. fatigue. incontinence)	0.0	14.7	0.054

Data indicated as mean (SD) or percentage.

Antimicrobial resistance and emerging pathogens

No ciprofloxacin or colistin resistance was observed in all initial infecting *P. aeruginosa* isolates. One isolate from both groups was aminoglycoside- and β-lactam resistant, respectively.

Enterobacteriaceae and Gram-positive bacteria were more frequently detected in cultures of the control group (table 3). The percentage of cultures containing non-fermenting Gram-negative bacteria excluding *P. aeruginosa* was significantly higher in the treatment group (p=0.0094). The presence of *Candida spp.* and fungi was not increased in the treatment group.

Table 3 Frequency of bacterial pathogens in respiratory cultures

	Control N	Treatment N	Control % (N=308)	Treatment % (N=368)*	Risk difference (95 % CI)	p-value
Gram-positive bacteria	189	188	61.36	51.09	10.3 (2.8;17.8)	0.007
Gram-negative bacteria	97	113	36.36	36.14	0.8 (-6.2;7.8)	0.830
Non-fermenting bacteria	24	52	7.79	14.13	6.4 (1.6;11.1)	0.009
Enterobacteriaceae	28	18	9.09	4.89	4.2 (0.39;8.0)	0.030
Other Gram-negative bacteria	45	43	14.61	11.68	2.9 (-2.2;8.0)	0.260
Yeast and fungi	152	190	49.35	51.63	2.3 (-5.3;9.8)	0.560

**Differences explained by the fact that in case of participants reaching the primary efficacy endpoint, data were analysed from the start of the study till the first full study year after having reached the endpoint.*

Discussion

This first prospective study into prophylaxis of initial *P. aeruginosa* infection by cycled antibiotic treatment failed to show a preventive effect in the entire group of children aged 0 to 18 years. Anti-*Pseudomonas* antibodies emerged later in the treated group but after three years this difference had disappeared.

Two previous observational retrospective studies[21;22] suggested a preventive effect of the use of anti-*Pseudomonas* prophylaxis. However, these studies were not controlled and the group of children twice daily treated with anti-*Pseudomonas* inhalation antibiotics was different from the group of untreated children. The approach in the current controlled trial was cycled treatment rather than daily treatment, to limit the patient burden and risk for antimicrobial resistance. Apparently, 3-monthly cycled therapy was not intense enough to prevent initial *P. aeruginosa* infection. In the first part of the study the survival lines of the two study groups diverged, indicating an emerging difference between the groups and postponement of infection in the treatment group. However after 1.5 year, a decline in the survival curve of the treatment group appeared leaving the survival curves more or less parallel. This difference in hazard ratios over time was not statistically significant. We could not find a clear reason for this steep decline, consisting of patients in all age categories. The reported compliance was not different. With respect to compliance, study intervention packages were not returned since it does not measure compliance. We wanted to reflect a 'real life' situation, and it is expected that in this situation compliance is not always optimal. Because of their own choice to participate, study participants are in general quite compliant.

Viral infections, especially the respiratory syncytial virus, predispose for pulmonary *P. aeruginosa* infection.[28] In this study, 13/19 (68 %) children acquired *P. aeruginosa* infection between the months October-March, suggesting a role for viruses. Continuous prophylaxis during the winter season or a cycle when experiencing a viral infection might better prevent for *P. aeruginosa* infections than cycled treatments over all the year. The number of exac-

erbations and antimicrobial agent use was not significantly different between the two groups. A possible reason for the lack in differences is that in young children viral infections often induce respiratory exacerbations, but antimicrobial treatment is not withheld because of the risk of bacterial infection. Recently, it was shown that children with CF often have more symptoms from viral respiratory infections in comparison with healthy controls.[29]
About 29 % of the patients in our study group reached the efficacy endpoint within 3 years; this meant an annual acquisition rate of about 11 %. The power analysis was executed assuming a 15 % annual acquisition rate and 75 % reduction in *P. aeruginosa* infections in the treated group. With a larger study group, it would have been possible to find smaller treatment differences. However in the group 0 to 5 years of age, the annual acquisition rate was 15 %. In this group, consisting of 24 patients, 38 % reached the primary endpoint but there was no difference between the control- and treatment group (hazard ratio 1.028; 95 % p=0.967). In the power calculation, we chose for 75 % reduction because smaller treatment differences would probably not justify the treatment burden and risks in comparison with regular culturing and intensive treatment of initial infection.

It is debatable whether the primary endpoint should be chronic infection. However, if prevention of initial infection occurs, inflammation and possible progression to chronic infection are arrested. Looking at definitions of chronic infection, patients from the control group showed elevated antibody titres, indicative of chronic infection, more early but the difference resolved after one year. The number of patients with chronic infection in the end of the trial was not different between the groups. Serological measurements were not very contributory in detecting initial infection. The timing of emergence of antibodies depends also on the antigens tested. Whole cell ELISA or type III system secretion products seem to be more sensitive than the antigens that were used in this study.[30;31] Notwithstanding, positive serology does not precede a positive culture in every patient.

Mannitol, an osmolytic agent, was used as control inhalation medication. At the time of study design, mannitol was not known as an airway clearance promoter for CF patients. Nowadays, clinical trials have been initiated to prove the effect of mannitol inhalation on pulmonary function and number of exacerbations.[32] It is not sure whether mannitol will have a preventive effect on acquisition of pathogens in the airways because of better airway clearance. Pulmonary function decline in a third group of patients (eligible patients that decided not to participate in the study) was estimated and was not different from the control group.
Intensive anti-*Pseudomonas* antimicrobial treatment might lead to selection of highly resistant bacteria such as *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*. The clinical importance of this finding is still unknown but should raise awareness among physicians.[33] Gram-negative non fermenting bacteria as a group (including *S. maltophilia*, *Acinetobacter spp*, and *B. cepacia* complex) were more frequently detected in cultures from the treatment group. *Candida* species were not more frequently detected in the treatment group, probably reflecting the overall high use of antimicrobial agents among CF patients.

Conclusion

Three-monthly cycled anti-*Pseudomonas* prophylaxis in *P. aeruginosa*-negative children with CF does not prevent initial pulmonary *P. aeruginosa* infection. Till now, frequent culturing and early intensive antimicrobial therapy when *P. aeruginosa* is detected is probably the best option especially in the light of treatment burden and emerging pathogens. Effort should be undertaken to identify those children or situations that imply additional risk for initial and chronic *P. aeruginosa* infection.

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Maintenance azithromycin treatment in paediatric cystic fibrosis patients: long-term outcomes related to macrolide resistance and pulmonary function.

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Abstract

Maintenance azithromycin therapy may improve pulmonary function in patients with cystic fibrosis (CF) with *Pseudomonas aeruginosa* infection because of its anti-inflammatory properties. However, azithromycin therapy might increase macrolide resistance in *Staphylococcus aureus* cultured from respiratory secretions. We studied the emergence of macrolide resistance in *S. aureus* and correlated this to pulmonary function decline in paediatric patients with CF on daily azithromycin therapy.

Respiratory cultures of one hundred patients with CF were analysed for *S. aureus* colonisation and its resistance pattern before and during 3 years after initiation of azithromycin maintenance therapy. Mean annual change in forced expiratory volume as percent of predicted forced expiratory volume in 1 second was calculated to compare pulmonary function before and after azithromycin therapy.

Staphylococcal colonisation did not significantly decrease after initiation of azithromycin (50 % v 48 %). Before start of therapy, 10 % of patients with staphylococcal colonisation had macrolide resistant strains. Staphylococcal resistance increased to 83 % in the first year; 97 % in the second and 100 % in the third year after initiation of azithromycin therapy ($p<0.001$). Half of macrolide resistant *S. aureus* comprised the macrolide-lincosamide-streptogramin (MLS)-phenotype. Percent forced expiratory volume in 1 second improved in the first year after initiation of azithromycin (mean annual change -4.75 % before v +3.09 % after initiation; $p<0.01$), but decreased during the second and third years after initiation (-5.15 % and -3.65 %, respectively). Emergence of macrolide resistant *S. aureus* was not related to pulmonary function decline.

Maintenance azithromycin therapy in patients with CF leads to macrolide resistance in nearly all *S. aureus* carriers. Pulmonary function improvement after initiation of azithromycin therapy seems to be temporary, and appears not to be related to macrolide resistance of *S. aureus*.

Introduction

Chronic pulmonary infections are the main determinant of morbidity and mortality in patients with cystic fibrosis (CF). *Staphylococcus aureus* and/or *Haemophilus influenzae* are responsible for most of the pulmonary infections in infancy. Chronic infection with *Pseudomonas aeruginosa* increases from 30 % in infancy to 80 to 90 % in adulthood. *P. aeruginosa* infection with biofilm-producing strains is associated with a strong decline in pulmonary function.[1]

Recently, macrolide antibiotics have been added to anti-*Pseudomonas* antimicrobial treatment in patients with CF with *P. aeruginosa* infection. Maintenance treatment with macrolide antibiotics had a beneficial effect in Japanese patients with diffuse panbronchiolitis, a disease that is also characterised by chronic pulmonary inflammation and *P. aeruginosa* infections.[2] The beneficial effect of macrolide antibiotics is probably related to its immunomodulatory properties. Macrolides have been shown to suppress inflammation, to interfere with bacterial virulence and decrease biofilm formation, unrelated to direct antimicrobial effects.[3]

Controlled trials showed discrete improvements in pulmonary function three months to one year after start of maintenance treatment with azithromycin in patients with CF.[4-7] Nevertheless, it remains unclear whether this improvement sustains beyond one year. Moreover, little attention has been paid to consequences of maintenance macrolide treatment such as emergence of resistant bacteria. From 1998 we introduced azithromycin in our CF centre on a regular basis in patients with chronic *P. aeruginosa* infection. From that time, we observed an increase of macrolide resistance among *S. aureus* isolates.

Macrolide resistance is determined by 2 mechanisms, namely by active drug efflux encoded by *mef* genes (M phenotype) or ribosomal target modifications by *erm* genes, which reduce macrolide affinity to the ribosomal target site. The latter is associated with lincosamide and streptogramin resistance as well because of overlapping binding sites on the ribosome (macrolide-lincosamide-streptogramin (MLS) phenotype).[8-10] Our aim was to determine macrolide resistance rates and mechanisms in *S. aureus* and relate this to pulmonary function decline in children with CF who were prescribed azithromycin maintenance therapy because of *P. aeruginosa* infection.

Materials and Methods

Participants

The study was performed in the CF centre of the Wilhelmina Children's Hospital, University Medical Centre Utrecht. All paediatric patients with CF that initiated maintenance azithromycin therapy between 1998 and 2005 were selected from our CF database. Dosage of maintenance azithromycin treatment was 5-10 mg/kg once daily. Other criteria for study inclusion were availability of at least one sputum or oropharyngeal swab culture before and after initiation of azithromycin therapy.

Microbiological and susceptibility analysis

Sputa or oropharyngeal swabs when sputum could not be expectorated by the patient were cultured on blood agar plates at 35°C for 18-24 hours. Bacterial identification and antimicrobial susceptibility testing was carried out with a BD PHOENIX automated microbiology system (Becton Dickinson Diagnostic Systems, Sparks, MD). Identification of *S. aureus* was confirmed by coagulase testing with rabbit plasma and by DNase testing; identification of *P. aeruginosa* was confirmed by oxidase testing. Macrolide susceptibility testing by the BD PHOENIX automatic system for *S. aureus* was confirmed by E-tests for erythromycin and clindamycin. All erythromycin-resistant, clindamycin-susceptible isolates were further tested for inducible clindamycin resistance by the double disk induction test with Rosco disks (Rosco Diagnostics, Taastrup Denmark). Clindamycin and erythromycin disks were placed approximately 20 mm apart on Mueller-Hinton agar supplemented with 5 % sheep blood and incubated for 18-24 hours at 35°C. A flattening of the clindamycin zone adjacent to the erythromycin disk was interpreted as a positive test, indicating that erythromycin resistance was of the MLS phenotype. One of the included patients was transiently colonised with methicillin resistant *S. aureus* (MRSA).

S. aureus and P. aeruginosa colonisation and resistance rates among the study group

The starting date of azithromycin treatment was established from the database and patient files. For every individual patient, the date of initiating azithromycin therapy was set as time point 0. Sputum or oropharyngeal swab cultures from the year before and every year after the start of azithromycin therapy were examined for presence of *S. aureus* and the macrolide resistance pattern. Patients were classified as *S. aureus* negative, macrolide susceptible *S. aureus* positive, macrolide resistant *S. aureus* (M-phenotype) positive and macrolide- and clindamycin resistant *S. aureus* (MLS-phenotype) positive. Resistance rates were calculated per patient, not per number of isolates.

Over the same time period, cultures were analysed for presence of *P. aeruginosa*, expression of mucoid phenotype, number of isolates, density and resistance against ciprofloxacin, polymyxin, aminoglycoside and β-lactam antibiotics. When ≥ 1 of the cultures in a year contained a mucoid *P. aeruginosa* and/or resistant phenotype, the patient was assigned to carry mucoid and/or resistant *P. aeruginosa* isolates during that subsequent year.

Pulmonary function

Pulmonary function was measured annually during routine follow-up visits. Forced expiratory volume in the first second (FEV_1) was measured according to criteria of the American Thoracic Society using the Master screen unit (Jaeger, Hochberg, Germany). FEV_1 was expressed as percent of predicted for sex and height (FEV_1 %). When more data were available per year, highest FEV_1 % was included in calculations. Annual change in FEV_1 % ($FEV_{1\,\%}\ year^{n+1}$ - $FEV_{1\,\%}\ year^{n}$) was estimated for every patient, starting the year before initiation of azithromycin therapy. Mean annual change in FEV_1 % was calculated to compare FEV_1 % change in time for the entire study group. In addition, the study group was divided in *S. aureus* and non-*S. aureus* carriers over the period after initiation of azithromycin to compare mean

annual change in FEV_1 % between the 2 subgroups.

Statistical analysis

S. aureus colonisation rates and macrolide resistance at different time points were compared with the χ^2 test for distribution of categorical variables (Statistical Package for Social Sciences version 12.0, Chicago, IL USA). A p-value <0.05 was regarded as significant. The change in FEV_1 % per time point and per patient subgroup was compared by using the 2-sided *t* test for paired samples.

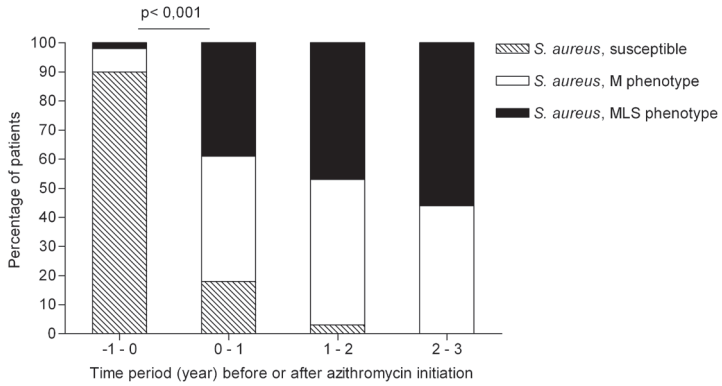
Results

From 1998, one hundred patients were given azithromycin as maintenance therapy. The mean age of the study group at time of therapy initiation was 10.8 years (SD 4.2; range 1.3-19.1 years), mean age at time of analysis was 14.4 years (SD 4.7; range 3.7-23.9 years). Mean duration of azithromycin use in this study group was 3.5 years (SD 1.6; range 0.9-7.0 years).

S. aureus colonisation decreased slightly but not significantly after azithromycin maintenance therapy (p>0.05). Fifty percent of patients were colonised with *S. aureus* before treatment, followed by 48 %, 44 % and 36 % respectively in the following first, second and third years. *S. aureus* colonisation was present in all age categories at time point 0 (43 % in the 3 to 12 years cohort *v* 51 % in the 12 to 21 years cohort).

Before azithromycin therapy, the macrolide resistance rate among patients with respiratory *S. aureus* colonisation was 10 % (figure 1). Following azithromycin initiation, macrolide resistance rates increased to 83 % in year 1 (p<0.001), 97 % in year 2 and 100 % in year 3. The M and MLS resistance phenotypes were approximately equally distributed following treatment; the MLS phenotype represented only 20 % of total resistance rate prior to therapy with an increase to 47 % in year 1, 52 % in year 2 and 56 % in year 3.

Figure 1 Emergence of macrolide resistance in *Staphylococcus aureus* after initiation of azithromycin maintenance therapy



Twenty-six percent of patients could not always provide sputum and did have occasionally or regularly an oropharyngeal swab taken for analysis of respiratory pathogens. The differences between sputum providers and non-sputum providers regarding presence of *S. aureus* and its macrolide resistance respectively are shown in table 1. In sputum, *S. aureus* was more often detected than in oropharyngeal swabs; the macrolide resistance rate among *S. aureus* cultured from the oropharynx did not differ significantly from the resistance rates of sputum isolates.

Table 1 Comparison of oropharyngeal swab cultures with sputa cultures with regard to presence of *Staphylococcus aureus* (indicated as percentage of the total number of oropharyngeal swabs or sputa) and macrolide resistance in *Staphylococcus aureus* (indicated as percentage of total number of *Staphylococcus aureus* in oropharyngeal swab and sputum group)

	Presence of *S. aureus*			Presence of macrolide resistance		
	Oropharynx	Sputum	p-value*	Oropharynx	Sputum	p-value
Before azithromycin initiation	37 %	59 %	0.096	0 %	13 %	0.309
Year +1	29 %	57 %	0.021	57 %	87 %	0.059
Year +2	18 %	53 %	0.011	100 %	97 %	0.744
Year +3	20 %	38 %	0.419	100 %	100 %	-

**calculated by χ^2 or Fisher's exact test*

The prevalence of *P. aeruginosa* and its mucoid expression among the study group increased slightly every year, which is shown in table 2. There was no significant difference observed in density and number of isolates. Resistance against aminoglycoside (tobramycin) and β-lactam antibiotics (piperacillin, ceftazidim) rose, whereas resistance against ciprofloxacin and polymyxins (colistin) remained stable. No patients carried *P. aeruginosa* isolates that were resistant against all anti-*Pseudomonas* antibiotics.

Complete pulmonary function data were available for 54 patients, with a mean age of 15.4 years (SD 3.7; range 7.4 to 23.9 years). Within this subgroup, 51 % was colonised with *S. aureus* before treatment initiation, followed by respectively 45 %, 37 % and 26 % in the first, second and third year after treatment initiation. The macrolide resistance pattern resembled the pattern of the complete study group.

Table 2 Prevalence of *Pseudomonas aeruginosa*, density, mucoid strains and resistance during the study period

		Before azithromycin initiation	Year 1	Year 2	Year 3
All participants					
Prevalence *P. aeruginosa*		63 %	69 %	78 %	80 %
Density > 100 cfu/agar plate		73 %	71 %	71 %	80 %
Patients with mucoid isolates		11 %	19 %	47 %	35 %
Resistance	*aminoglycoside*	0 %	10 %	14 %	20 %
	ciprofloxacin	15 %	13 %	12 %	16 %
	β-lactam	13 %	27 %	24 %	37 %
	polymyxin	0 %	0 %	2 %	0 %
Participants with pulmonary function data					
Prevalence *P. aeruginosa*		66 %	78 %	84 %	79 %
Density > 100 cfu/agar plate		81 %	64 %	79 %	76 %
Patients with mucoid isolates		6 %	13 %	52 %	49 %
Resistance	*aminoglycoside*	0 %	8 %	12 %	21 %
	ciprofloxacin	13 %	10 %	7 %	15 %
	β-lactam	13 %	21 %	24 %	42 %
	polymyxin	0 %	0 %	0 %	0 %

Data on *P. aeruginosa* colonisation in this subgroup are indicated in table 2. In the year after treatment initiation, a significant difference in mean annual change in FEV_1 % was observed ($p < 0.01$; 95 % CI -13.8 to -2.4). Figure 2a shows mean changes in FEV_1 % with 95 % confidence intervals. In year 2 and 3, mean FEV_1 % decreased again. Comparison of annual change in FEV_1 % between patients with and patients without *S. aureus* per time point revealed no significant differences (figure 2b).

Figure 2a Mean annual change (with 95 % confidence interval) in FEV_1 % before and after initiation of azithromycin treatment (N=54)

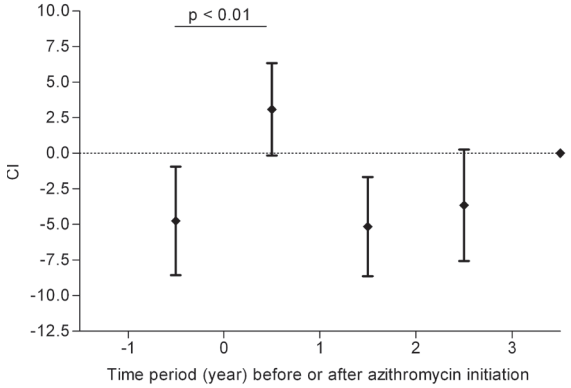
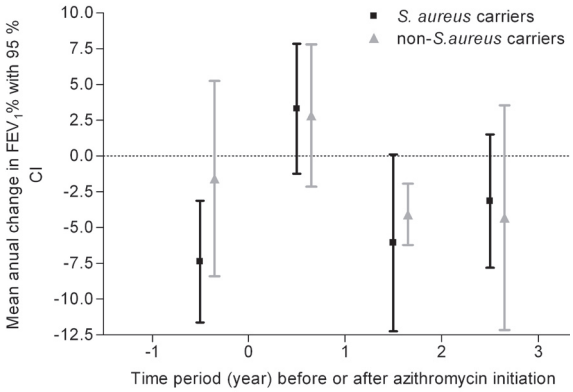


Figure 2b Mean annual change (with 95 % confidence interval) in FEV_1 % divided by *Staphylococcus aureus* colonisation (N=2 x 27)



Discussion

We described the emergence of macrolide resistance rates in *S. aureus* in the first year and following years after introduction of maintenance azithromycin therapy in patients with CF. Both the efflux (M) and ribosomal (MLS) phenotype were approximately equally distributed. An improvement in pulmonary function appeared to be only a temporal outcome of azithromycin therapy.

A dramatic rise of macrolide resistance in *Streptococcus pneumoniae* and other streptococci has been observed over the last decade related to the increased prescription of macrolide antibiotics for community acquired respiratory infections.[9;10] Moreover, macrolide resistance in staphylococci has increased. A follow-up study in children demonstrated macrolide resistance in *S. aureus* in 72 % of isolates 1 to 6 weeks after a 3-day azithromycin course.[11] In a randomised controlled trial with 296 patients, 14-day clarithromycin therapy induced significantly more macrolide resistance in staphylococci compared with placebo; this resistance sustained for at least 8 weeks after therapy.[12]

In our study population, resistance rates increased to 83 % in the first year after initiation of azithromycin. This indicates that resistance emerges soon after antibiotic pressure increases, a phenomenon that is confirmed by the studies cited here in which macrolide resistance was observed shortly after a single macrolide course.

Acquisition of resistance can be explained by different mechanisms. Selection of *S. aureus* strains that previously acquired resistance and induction of new mutations after antibiotic pressure can both be responsible for expanded resistance. Patients with CF are known to have high proportions of hypermutable *S. aureus* strains, probably as a result of continuous antibiotic pressure.[13] In our hospital, surveillance of clinical *S. aureus* isolates in patients without CF during the years 2002 through 2005 shows that 9 % of patients positive for *S. aureus* are colonised with macrolide-resistant strains (Dr. M. Visser, Department of Medical Microbiology, personal communication). These data are comparable with the macrolide resistance rate among the CF study group before azithromycin therapy. Clinical surveillance

data from patients without CF demonstrate 56 % M phenotype and 44 % MLS phenotype among macrolide resistant *S. aureus*. The resistance phenotypes in the CF study group have approximately the same distribution. Clonal spread of macrolide resistant *S. aureus* among household members or other close contacts of the index patient should be investigated. Dissemination of resistant strains might in case of MLS phenotypes be a problematic situation because treatment options of MLS resistant *S. aureus* infection are restricted, especially in the light of methicillin coresistance.

Noteworthy is the fact that in the majority of patients staphylococci do not disappear with daily azithromycin treatment, probably because of resistance induction, a finding that was also described by Saiman *et al.*[6] Pulmonary *S. aureus* colonisation was demonstrated in all age groups from 3 to 21 years old. *S. aureus* was more often detected in sputum providers, which might reflect the low sensitivity of oropharyngeal swabs for lower respiratory tract pathogens. However, we tried to reduce the chance of false negative classification of patients with oropharyngeal swabs by more regular culturing. Increasing resistance to first-line antibiotics among clinical isolates of *S. aureus* might add to the burden of respiratory infections in patients with CF, because treatment fails to eliminate the microorganism from the respiratory tract.

Pulmonary function data do not support this suggestion. As reported by others, in our study group, annual pulmonary function decline changed into pulmonary function improvement in the first year after start of azithromycin.[5-7] The mean annual change in FEV_1 % in the year after azithromycin therapy differed significantly in a positive way from the FEV_1 % change before therapy. However, no sustained positive effect on FEV_1 % after one year on pulmonary function was seen in our study group. Pulmonary function declined after 1 year of maintenance therapy with approximately the same rate as before therapy. To our knowledge, there are no other data that describe pulmonary function outcome following azithromycin treatment beyond one year. The long-term effects of azithromycin on pulmonary function in our study must be interpreted with caution, because we did not perform a prospective, controlled trial. However the timing of emergence of macrolide resistance and the improvement in pulmonary function in the first year strongly argues that both phenomena are unrelated. This is further supported by the fact that pulmonary function improves in the first year and declines in subsequent years in both *S. aureus* colonised and -noncolonised patients. Emerging resistance in staphylococci could not explain the decline of pulmonary function after first treatment year. An increase in *P. aeruginos*a prevalence was observed in year 1 and 2 after azithromycin initiation; no difference in prevalence between year 1 and year 3 was seen (table 2). As a consequence this could not explain pulmonary function decline after the first year. Nevertheless, percentage of mucoid isolates and resistance against ?-lactam and aminoglycoside antibiotics increased, which might explain further lung function decline despite macrolide maintenance treatment. Although the increase in mucoid isolates was also observed in the first year after treatment initiation, it was more pronounced in the second and third year. We could not compare percentage of mucoid phenotypes in isolates of patients that did not take regular azithromycin, since nearly all patients with chronic *P. aeruginosa* infection were prescribed azithromycin. Hansen et al described the prevalence of a mucoid phenotype in 90 % of isolates before start and in 81 % 1 year after azithromycin initiation.[7] Overall, the prevalence of mucoid isolates in our study cohort is lower (52 % of patients carried ever a mucoid isolate during year 2). No multidrug resistance in *P. aerugi-*

nosa was observed in the study group, all isolates analysed were at least susceptible to 2 anti-*Pseudomonas* antibiotics. β-lactam and aminoglycoside resistance reflects our treatment policy of *P. aeruginosa* infections in patients with CF.

In conclusion, we have shown a dramatic rise in macrolide resistant *S. aureus* in patients with CF using maintenance macrolide antibiotics to modulate *P. aeruginosa* infection. This resistance comprises approximately 50 % MLS phenotypes. Pulmonary function seems to improve only short term after start of macrolide maintenance treatment. During the second and third years, pulmonary function declines again, but this is not related to resistance of staphylococci and increase of *P. aeruginosa* prevalence. The clinical relevance of emerging macrolide resistance in *S. aureus* for patients with CF and the community and the long-term course of pulmonary function after azithromycin initiation warrant further study.

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Boy selling sweets - Rajasthan, India

8

Macrolide resistant *Staphylococcus aureus* colonisation in cystic fibrosis patients: is there transmission to household contacts?

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Abstract

Patients with cystic fibrosis (CF) are frequently colonised by macrolide-resistant *Staphylococcus aureus*, a result of maintenance macrolide therapy. As transmission of *S. aureus* between household contacts is common, we examined the prevalence of macrolide-resistant *S. aureus* colonisation in CF patients on maintenance azithromycin therapy and their household contacts and compared this with the *S. aureus* macrolide resistance prevalence in the community.

Sixty-five CF patients on maintenance macrolide therapy and 194 household contacts were screened for *S. aureus* colonisation by culturing sputa, cough swabs and nasal swabs. Resistance to macrolide, lincosamide and methicillin was determined by disc diffusion tests. The prevalence of macrolide-resistant *S. aureus* colonisation in both groups were compared with figures from a nationwide study into *S. aureus* carriership and resistance. To assess possible transmission, genotyping of *S. aureus* was performed using the *spa*-typing method.

Macrolide resistance among CF patients with *S. aureus* colonisation was 69.6 %, 75 % of these isolates displayed lincosamide resistance too. Among household contacts, macrolide resistance prevalence did not differ significantly from resistance prevalence in the community (9.6 % *v.* 6.3 %; p=0.358). No methicillin resistance was observed. No identical (macrolide resistant and -susceptible) *S. aureus* genotypes were observed between CF patients and their household contacts except for one household, suggesting a probable transmission.

No significant increase in macrolide-resistant *S. aureus* colonisation was observed among household contacts of CF patients on long-term azithromycin therapy. Transmission of macrolide-resistant *S. aureus* could not be proved by genotyping in the majority of households.

Introduction

Macrolide resistance of frequently colonising pathogens is clearly associated with prescription of macrolide antibiotics.[1;2] A subgroup of patients who frequently use macrolides are cystic fibrosis (CF) patients. These antibiotics are mainly prescribed for CF patients because of their anti-inflammatory properties. Additionally, macrolides reduce *Pseudomonas aeruginosa* biofilm formation and inhibit *P. aeruginosa* quorum sensing in-vitro.[3] Placebo-controlled trials showed benefit with relation to frequency of exacerbations and pulmonary function after 6-9 months of macrolide treatment.[4] Nevertheless, little attention is paid to long-term effects of macrolide use. Our and other groups previously reported a very high rate of macrolide-resistant *Staphylococcus aureus* colonisation in CF patients on long-term azithromycin therapy.[5;6]

The resistance induced by macrolide use appears to be rapidly emerging and long-lasting, even after a short-course therapy.[7;8] Macrolide-resistance in *S. aureus* is characterised by drug efflux and prevention of drug binding on the ribosomal target site, induced by *mef* and *erm* genes, respectively. Cross-resistance to clindamycin and streptogramin$_B$ is observed with the latter mechanism.

S. aureus often colonises the respiratory tract of CF patients, but also approximately 30 % of the healthy population are nasal *S. aureus* carriers.[2] Transmission of *S. aureus* is frequent among household contacts, including in families with CF patients.[9] *S. aureus* colonisation may have significant impact, since nasal *S. aureus* colonisation is known to be a strongly associated risk factor for subsequent *S. aureus* infection.[10;11]

We hypothesised that if *S. aureus* transmission is frequent among household contacts, macrolide-resistant *S. aureus* from CF patients can spread into the community via household contacts. The same phenomenon has been observed for methicillin resistant *S. aureus* colonisation.[12] The prevalence and transmission of macrolide-resistant *S. aureus* colonisation might have consequences for the empiric treatment of *S. aureus* infections, especially in the light of cross-resistance to clindamycin.

To assess transmission of macrolide-resistant *S. aureus*, we examined household contacts of CF patients on maintenance macrolide therapy for nasal *S. aureus* carriage and subsequent macrolide resistance and compared these figures with nationwide surveillance data of *S. aureus* nasal carriage and resistance rates.

Patients and Methods

Participants and material collection

CF patients are routinely seen at the outpatient clinic of the Wilhelmina Children's Hospital, where they regularly provide sputa or cough swabs. All paediatric CF patients on maintenance azithromycin therapy (5-10 mg/kg/d) and their household contacts were asked to participate. The study was approved by the hospital ethical review board. Sixty-five CF patients with 193 household contacts (adults and children) provided informed consent. Participants were screened for nasal *S. aureus* colonisation by culturing nasal swabs. They were instructed to firmly rotate swabs in both the anterior nares; half of the participants repeated

this three times with intervals of 2 hours.
Nasal swabs from the community were obtained from 2641 patients visiting their general practitioner for a non-infectious complaint. The general practitioners participated in the NIVEL sentinel station project and were located all over the Netherlands.[13]
A questionnaire about antibiotic use in the previous 2.5 years was obtained from household contacts.

Microbiology

Nasal swabs were cultured onto 5 % sheep blood agar (household contacts) or mannitol salt agar (CF patients). After 24 hours, all morphological different colonies with features of *S. aureus* were purified on blood agar plates and identification was performed with catalase, tube coagulase and DNase testing. Resistance to macrolides, lincosamides and methicillin was determined by disc diffusion tests on Mueller-Hinton agar, inoculated with a bacterial suspension adjusted to an optical density of 0.5 McFarland standard. Neo-Sensitabs (Rosco, Taastrup, Denmark) containing erythromycin, clindamycin and oxacillin were placed on the Mueller-Hinton agar with the inoculum. Clinical Laboratory Standards Institute (CLSI) guidelines for resistance cut-off levels were used to classify the isolates. A study subject was considered colonised with macrolide resistant *S. aureus* if one or more of the colonies were macrolide resistant.
To assess possible transmission between household contacts, genotyping was performed with the staphylococcal protein A (*spa)*-typing method, as described previously.[14] Only isolates from CF patients carrying macrolide-resistant *S. aureus* having household contacts with *S. aureus* colonisation (macrolide-resistant and non-resistant) were typed. A dendrogram was created and patterns were assigned identical when there was 100 % concordance in sequence type.

Comparison with control group

Prevalence of *S. aureus* colonisation among household contacts and macrolide resistance was compared with figures from the nationwide study into *S. aureus* nasal colonisation and resistance. Differences in prevalence were estimated with the χ^2 test or Fisher's exact test, and calculated with SPSS version 12.0 (IL, USA)

Results

The prevalence of *S. aureus* colonisation among CF patients was 35.4 % (table 1). Nasal prevalence was lower than sputa prevalence. Of CF staphylococcal carriers, 31 % carried *S. aureus* in both nose and sputum/cough swab, 17 % only had a positive nose swab and 52 % had a single positive sputum/cough swab without concomitant positive nose swab. Prevalence of nasal colonisation among 193 household contacts was 26.9 %. There was no difference in *S. aureus* detection percentages after taking one nasal swab versus three nasal swabs with 2-hour intervals (25.5 % *v.* 28.4 %; p=0.649).

Table 1 Prevalence of *S. aureus* colonisation, macrolide resistance in CF patients, household contacts and a control group

	CF patients (N=65)	Household contacts (N=193)	Population (N=2641)	p-value
Total *S. aureus* colonisation	23 (35.4 %)	52 (26.9 %)	663 (25.1 %)	
Nasal	11/63 (17.5 %)	52	663	
Cough swab/sputum	19/64 (29.7 %)	-	-	
Macrolide resistance	16 (69.6 %)	5 (9.6 %)	42 (6.3 %)	0.358*
Only macrolide	4 (25.0 %)	1 (20.0 %)	17 (40.5 %)	
Macrolide and clindamycin	12 (75.0 %)	4 (80.0 %)	25 (59.5 %)	
constitutive	10	2	-	
inducible	2	2	-	
Methicillin resistance	0 (0 %)	0 (0 %)	5 (0.75 %)	

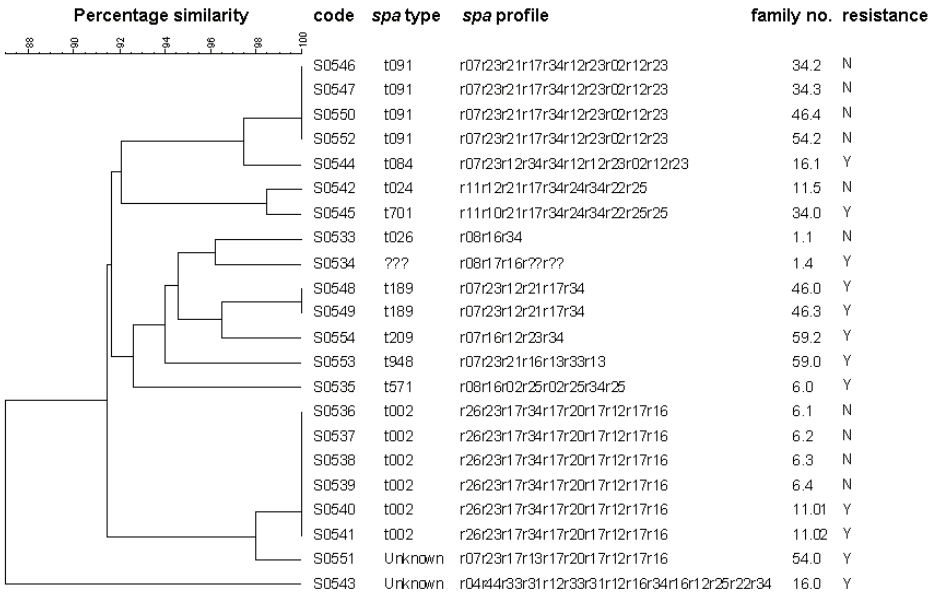
**χ^2 test, household contact v. population*

Macrolide resistance among CF *S. aureus* was 69.6 %; 75 % of these isolates were inducible or constitutive clindamycin resistant. Prevalence of macrolide-resistant *S. aureus* among household contacts was not significantly higher than in the control population (9.6 % *v.* 6.3 %; p=0.358). No methicillin-resistant *S. aureus* was detected among CF patients and household contacts.

Macrolide use among household contacts with macrolide-resistant *S. aureus* in the previous 2.5 years was 40 %; use among household contacts with macrolide susceptible *S. aureus* 8 % (Fisher's exact test p=0.087; odds ratio 8.2 (0.966-69.9)). All five household contacts colonised with macrolide-resistant *S. aureus* shared the household with a CF patient colonised by macrolide-resistant *S. aureus* either continuously or intermittently during last year.

Spa-genotyping (figure 1) demonstrated sharing of isolates between non-CF household contacts (non-CF siblings and parents, no. 6, 11, 34). No sharing of isolates within one household between CF patients and non-CF contacts was revealed except for one household (no. 46), probably indicating transmission of macrolide resistant *S. aureus* between CF patient and household contact.

Figure 1 Dendrogram of *spa*-typing



Every number represents one household contact, .0 indicates CF patient; .1-.5 indicates household contact (non-CF siblings and parents). Y=yes; N=no.

Discussion

We did not observe an increased prevalence of macrolide resistant *S. aureus* colonisation among a large group of household contacts of CF patients with maintenance macrolide use and regular hospital visits. On the basis of risk factor data from methicillin-resistant *S. aureus* prevalence studies,[12] we expected an increased prevalence of macrolide-resistant *S. aureus* in household contacts of CF patients regularly.

These findings suggest that transmission of macrolide-resistant *S. aureus* or mobile genetic elements carrying resistance genes from CF patients to household contacts is limited. *Spa*-genotyping demonstrated no concordance between CF and household isolates in all households except one. Accordingly, we did not observe identical resistant and non-resistant isolates within one household. The latter might occur when resistance is lost after transmission and antibiotic pressure has disappeared in the household contact. However, we did not expect this because expression of macrolide resistance genes in staphylococci is able to last for a prolonged time in the absence of continuous antibiotic pressure and continuous contact occurs between CF patients and household contacts.[7;8] In contrast, transmission

from CF patients to household contacts may likewise occur, as was proved in case 46 (figure 1). Yet in the majority of household contacts, bacterial competition with susceptible *S. aureus* isolates might protect for colonisation with resistant isolates, as was described for methicillin-resistant *S. aureus*.[15]

We did not investigate transfer of mobile genetic elements as the prevalence among household contacts was not significantly higher than in the control population. In addition, colonisation with macrolide-resistant *S. aureus* was associated with macrolide use in the previous 2.5 years in household contacts.

Some small contrasts exist between the study and control groups. The prevalence of macrolide-resistant *S. aureus* colonisation in the nationwide study was somewhat lower, however not significant. This control group mainly consisted of adult participants while in the present study, the majority of household contacts were children. Staphylococcal colonisation rates are higher in young children; additionally young children more frequently experience respiratory infections, thus having a higher a priori chance of previous macrolide antibiotic therapy.[10] Clinical *S. aureus* isolates from our hospital are macrolide resistant in approximately 9 to 10 %, corresponding to the prevalence in our study group.

The prevalence of nasal *S. aureus* in CF patients was lower than among household contacts and another CF group previously described. Goerke *et al.* estimated nasal colonisation in 29 % of CF patients with antibiotic use in the previous 4 weeks; 66 % of patients without antibiotic use were colonised.[9] Probable reasons for the lower prevalence in our study group are long-term exposure to antibiotic treatment and presence of chronic respiratory *P. aeruginosa* colonisation, competing with *S. aureus* colonisation.

The present outcome of macrolide-resistant *S. aureus* prevalence in household contacts differs from the prevalence in other parts of the world. In 2 studies from the USA, a prevalence of 26 % in a student community and 32 % in a more open population was observed.[2;10] The contrasts can be attributed to a very low frequency of methicillin resistance and a limited antibiotic usage. Both are the result of effective control of nosocomial infections and strict antibiotic policy in the Netherlands.

In conclusion, no increased colonisation with macrolide-resistant *S. aureus* could be established among household contacts of CF patients on maintenance azithromycin therapy. From these data, it seems that antibiotic use is a higher risk factor for macrolide-resistant *S. aureus* colonisation than transmission from a macrolide-resistant *S. aureus* carrier.

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Girls preparing for ceremonial dance - Ubud, Indonesia

9 Summarising discussion and future research directions

Summarising discussion

Pulmonary infections in children with CF are a major cause of morbidity and mortality. In particular, infections with *P. aeruginosa* lead to rapid pulmonary function decline and are hard to eradicate once established and adapted to the host environment. In this thesis, determinants of host- and bacterial factors for *P. aeruginosa* infections were investigated. Because of the possible benefit of early detection of *P. aeruginosa* with respect to eradication strategies, the diagnostic value of direct and indirect detection methods was examined. Also, interventions directed at prevention of initial *P. aeruginosa* infection by cycled antibiotic prophylaxis and directed at prevention of further pulmonary decline after establishment of *P. aeruginosa* infection by macrolide antibiotics were analysed.

The major findings of this thesis are:

- In CF pulmonary infection, the genetic diversity of *P. aeruginosa* isolates is lower than in *P. aeruginosa* isolates from infections in non-CF hosts. Clonal genotypes were observed in Dutch patients with CF, but not in patients without CF (chapter 2).
- In CF, both eradicated and persistent initial colonising *P. aeruginosa* isolates show highly variable bacterial characteristics. It is not possible to predict persistence in the airways after initial *P. aeruginosa* colonisation by estimating these bacterial characteristics alone (chapter 3).
- Increasing the culture frequency of oropharyngeal swabs enhances the diagnostic values for *P. aeruginosa* presence in the lower airways (chapter 4).
- Serological measurements of specific anti-*P. aeruginosa* antibodies are sensitive in detecting chronic *P. aeruginosa* infections but do not yet contribute substantially in early detection of *P. aeruginosa* colonisation in the majority of children (chapter 4, 5 and 6).
- Cycled 3-monthly anti-*Pseudomonas* antibiotic prophylaxis does not prevent initial and persistent *P. aeruginosa* infection and subsequent lung function decline in children with CF (chapter 6).
- Azithromycin therapy leads to a temporary pulmonary function improvement, but induces rapid macrolide resistance in *S. aureus* without significant decline in S. aureus isolation. Transmission of macrolide-resistant *S. aureus* to household con tacts of patients with CF was not proven (chapter 7 and 8).

Determinants

The majority of patients with CF will experience pulmonary *P. aeruginosa* infection. Some acquire *P. aeruginosa* early in life, whilst others only in adolescence or adulthood. Nowadays, first *P. aeruginosa* can often be eradicated with intensive antimicrobial therapy. Nevertheless, some patients rapidly continue to persistent, chronic infection. To unravel characteristics of *P. aeruginosa* that might predispose to eradication failure and chronic infection, *P. aeruginosa* from CF patients and non-CF patients were analysed for phenotypic and genotypic characteristics. The bacterial genotypes of initial colonising isolates were highly diverse

(chapter 2 and 3). A clonal genotype was observed among approximately 50 % of CF patients with chronic infection. Interestingly, in immunocompetent and immunocompromised non-CF patients (n=101) with diverse other *P. aeruginosa* infections (chronic suppurative otitis media, urinary tract infection, sepsis or severe pulmonary infection (admitted on the ICU)), this specific clonal genotype was not observed. This suggests a common source and/or CF host susceptibility for this bacterial genotype. The ability of the clonal genotype to induce chronic infection of the CF airways was proved by the fact that in two CF patients with initial colonisation, the infection persisted even after intensive antimicrobial treatment. In other countries, CF clonal isolates have been observed among non-CF patients and even domestic pets; these clonal isolates possessed increased virulence and/or multidrug resistance. In contrast, the limited investigations of the Dutch clonal genotype described in this thesis, point to a decreased expression of virulence factors and absence of multidrug resistance (chapter 2). Attenuated virulence might explain this clone not being observed among non-CF patients with *P. aeruginosa* infection. The degree of virulence alone however, does not explain the success of persistence in CF patients. Among initial colonising non-clonal isolates that persisted in the CF airways, a large variability in bacterial virulence was observed (chapter 3). A predefined set of bacterial characteristics was measured, and compared to the characteristics of eradicated isolates. The outcome of the comparative analysis showed no clear distinction between the two groups and pointed out that bacterial mechanisms for persistence are still largely unknown. Success of persistence in patients with CF will sometimes be defined by the bacterium (in case of the successful clonal genotype) but most often be defined by the complex host-pathogen interaction. Within the studied group, higher age was the only discriminating host factor associated with persistence (chapter 3). At the same time, the *P. aeruginosa* infection process is influenced by co-colonisation with other microbial organisms, *eg.* the respiratory syncytial virus. Microbial interaction probably plays a significant role as proven by some studies into up- and downregulation of important *P. aeruginosa* genes. Most probable, a complex interplay between host, bacteria and other microorganisms renders some patients with CF extra prone for rapid progression to chronic *P. aeruginosa* infection.

Detection

The detection sensitivity of *P. aeruginosa* relies on both the way of sample providing as well as on microbiological techniques. Oropharyngeal and/or cough swab cultures have lower diagnostic values for pulmonary *P. aeruginosa* infection compared to sputum cultures, which might lead to a delayed detection of *P. aeruginosa*. These values increase when samples are taken more frequently (chapter 4). However, possible delayed detection because of swab culturing instead of sputum culturing was not associated with persistent infection after initial colonisation, even when corrected for age (chapter 3). Data from the prospective study (chapter 6) show that the majority of positive oropharyngeal swab cultures were followed by a subsequent positive swab culture. This fact justifies the initiation of eradication therapy after a single positive swab culture, despite the suboptimal positive predictive values for *P. aeruginosa* in the lungs. Sputum induction by inhalation of hypertonic saline did not lead to a significant extra diagnostic yield (chapter 4) and is therefore not routinely advised.
Till now, culture has been the gold standard for *P. aeruginosa* detection. Traditional detec-

tion by culture is prone for misidentification since the *Pseudomonas* genus exists of many species that are difficult to discriminate. This is overcome by molecular techniques which are more sensitive and specific to detect *P. aeruginosa* in respiratory specimens. Another major advantage of molecular techniques is the possibility to apply specific primers to detect clonal types, providing insight into cross-infections. Other techniques such as mass spectrometry are promising but need to be further developed for clinical practice.

The contribution of serological measurements to indirectly diagnose initial *P. aeruginosa* infection before direct diagnosis by culture has been extensively studied but is not conclusive (chapter 4). Serological tests based the detection of antibodies directed to *P. aeruginosa* virulence factors depend on the expression of those factors. These tests perform fairly well to detect and monitor chronic infection. However, a limited number of patients show positive serology before a first positive culture, as shown in the review and original research (chapter 4, 5, 6). Serological tests against other antigens which are expressed more early in the infection phase might perform better compared to the antigens used in these studies. In different studies, positive serology is observed years before a positive culture, leading to the question whether any action should be initiated. It is also unclear whether these anti-*Pseudomonas* antibodies have affinity for the first detected *P. aeruginosa* in respiratory specimens.

Before implementing anti-*Pseudomonas* serology as a frequent standard measurement to trace initial infection, consequences for treatment policy must be discussed taking into account the physical and psychological burdens for patients. Besides, it is unclear whether patients with eradication failure after initial infection would have benefited by earlier detection. In the light of the prospective trial (chapter 6), regular eradication therapy in culture-negative patients did not lead to prevention of initial *P. aeruginosa* infection in a later stage. Patients with positive serological tests at the trial entry who initiated cycled prophylaxis (but were excluded from the trial analysis) did not achieve eradication after experiencing their first positive culture. Another patient with positive serological tests at trial entry taking control placebo treatments achieved eradication after first positive culture and subsequent treatment. Also in this case, the complex host-pathogen interaction is probably more important than a single serological indicator.

Directed interventions

It was hypothesised that early *P. aeruginosa* infection would be prevented or eradicated by prophylactic therapy before its detection. Despite, the *P. aeruginosa* infection rate did not significantly decrease among the prophylactic intervention group (chapter 6). In addition, cycled prophylaxis did not decrease the risk for persistent infection. The intensity of the prophylactic schedule might be too low to prevent for infections in between the intervention periods. Also, pulmonary function decline was not arrested by cycled prophylaxis. Importantly, prophylaxis led to a shift in bacterial colonisation with increased isolation of intrinsically resistant gram-negative bacteria and less frequent culturing of gram-positive organisms, which might lead in the end to a worse situation. Increasing the frequency of prophylactic cycles or increasing the number of weeks on prophylaxis will probably lead to more microbiological adverse effects. Therefore, the focus with respect to prophylaxis must be directed at specific conditions that predispose for acquiring *P. aeruginosa* infection in the already susceptible patient with CF. Segregation measures are already implemented to prevent cross-infections with adapted *P. aeruginosa*, such as the clonal genotype, from other patients with CF. It is still

unsure whether segregation measures prevent chronic infections in the whole CF cohort.

Adverse effects related to regular antimicrobial agent use were also noted with maintenance azithromycin treatment (chapter 7). Macrolide resistance in *Staphylococcus aureus*, often combined with clindamycin resistance, rose steeply after initiation of azithromycin. Increasing resistance did not lead to a decline in pulmonary function; in contrast the pulmonary function temporarily improved. The major reason for prescription of azithromycin was persistent *P. aeruginosa* infection. The presumed actions of azithromycin are related to interaction with host inflammation and bacterial virulence. These mechanisms are proven in in-vitro models, but long-term clinically relevant findings with respect to *P. aeruginosa* are not yet available. It remains unclear whether more early initiation of azithromycin treatment just after the first colonisation might have a favourable effect on *P. aeruginosa* eradication, despite its capacity to rapidly induce resistance. Fortunately, dispersion of macrolide resistant *S. aureus* to the community seems to be limited (chapter 8) in contrast with methicillin resistant *S. aureus* from patients with CF.

Directions for future research

Determinants

Clonal isolates, occurring worldwide among CF populations, are successful colonisers of CF airways. Therefore, effort must be made to unravel the specific pheno- and genotypic characteristics of clonal isolates related to their success. This could also be of importance to unravel infection potential for other non-clonal *P. aeruginosa* isolates and might lead to development of more specifically directed therapies to prevent chronic infection.
Next, initial infections should be monitored more closely, especially with respect to the occurrence of other micro-organisms such as viruses. Pathogen-pathogen interactions should be studied on an epidemiological but also molecular basis to reveal important interactions that might promote persistence of *P. aeruginosa* in the CF airways. The role of viruses in pathogenicity and cytotoxicity of *P. aeruginosa* should be investigated in in-vitro and in-vivo models. Also, the host innate immune response to *P. aeruginosa,* for example related to Toll-like receptor 5, should be further elucidated to identify those patients at risk for impairment of bacterial clearance.

Detection

Detection is a prerequisite for possible eradication by intensive antimicrobial treatment. Therefore, highly sensitive and specific methods should be implemented in CF care. Additionally, these tests must be rapid and not labour intensive in contrast to current culturing techniques. Innovative multiplex PCR assays or chips with 16s rDNA primers designed to detect micro organisms relevant for CF patients will lead to more rapid and highly sensitive determination. Also, interactions with other species can be studied since with these techniques it is possible to estimate the colonisation with several micro organisms at a certain time.

The place of serological measurements before direct detection of *P. aeruginosa* should be estimated. Therefore, more sensitive and specific antigens should be determined to improve the reliability. Especially the consequences of positive serology with respect to treatment must be discussed. Picking those CF patients at increased risk for rapid chronic infection without overtreating others will be a major challenge.

Directed intervention

Since cycled prophylaxis does not prevent initial infection in a large group of children, specific situations should be designated in where prophylaxis might be of more benefit. Examples are seasonal prophylaxis during the viral (winter) season or prophylaxis during viral infections and/or respiratory signs and symptoms. Age should be taken into account, since a higher age was associated with development of chronic infection after initial colonisation. More specific host risk factors, *eg.* based on immunity profiles, should be defined. Then it can be estimated whether patients with these risk factors will benefit from prophylaxis.

Additionally, the value of adjuvant therapies next to antimicrobial therapy to eradicate *P. aeruginosa* infection in an early stage should be investigated in a controlled setting. Medicaments that focus specifically on bacterial exoproducts and/or the quorum sensing system should be developed. Macrolide antibiotics seem to have some quorum sensing inhibiting potential but also have other mode of actions; their contribution in eradication of initial *P. aeruginosa* infections should be estimated. Other promising therapies are inhaled anti-oxidants reducing the excess of oxygen radicals created by release of *P. aeruginosa* pyocyanin. These new possible therapies should be investigated in a randomised controlled manner taking into account safety, efficacy and optimal dose.

Girl - Taroudant, Morocco

10

Nederlandse samenvatting

Nederlandse samenvatting

Introductie

Cystic fibrosis (CF) is autosomaal recessieve ziekte veroorzaakt door mutaties in het cystic fibrosis transmembrane regulator (CFTR) gen op chromosoom 7, en komt in Nederland bij ongeveer 1 op de 4750 pasgeborenen voor. Afwijkend CFTR leidt tot een verstoorde regulatie van zout- en watertransport over de celmembraan. Aangedane organen zijn met name de luchtwegen, het maag-darmstelsel en het reproductieve systeem. Luchtweginfecties zijn een belangrijke oorzaak voor morbiditeit en mortaliteit bij patiënten met CF. Deze infecties worden onder andere veroorzaakt door de bacteriën *Staphylococcus aureus* en *Pseudomonas aeruginosa*. Vooral (chronische) infectie met *P. aeruginosa* heeft een sterk negatieve invloed op de longfunctie. De kans op het krijgen van een chronische infectie met *P. aeruginosa* neemt toe met de leeftijd, waarbij zonder tijdige behandeling ongeveer 80 % van alle patiënten met CF geïnfecteerd zullen raken.

Er zijn een aantal hypotheses waarom een patiënt met CF gevoelig is voor infecties met *P. aeruginosa*. Door een afwijkende productie en transport van vloeistof in de longen is er meer stase van bacteriën. Daarnaast is er een hoge zoutconcentratie in de longen waardoor lichaamseigen antimicrobiële eiwitten verminderd actief zijn. Een toegenomen binding aan de celmembraan door specifieke receptoren op de celmembraan lijkt mede infectie met *P. aeruginosa* te faciliteren. Daarnaast spelen bacteriële factoren tevens een belangrijke rol. *P. aeruginosa* heeft een sterk aanpassend vermogen. Voorbeelden hiervan zijn het vormen van een biofilm, waardoor de bacteriën relatief beschermd zijn voor invloeden van buitenaf, zoals een antibioticum. Virulentiefactoren (bacteriële producten welke gericht zijn tegen de gastheer) worden veelvuldig uitgescheiden door *P. aeruginosa*. De uitscheiding van virulentiefactoren kan echter wisselen en de bacterie kan zich op die manier aanpassen aan de gastheeromgeving. Vanwege de chronische aard van een infectie met *P. aeruginosa* en de gevolgen is het belangrijk om deze te voorkomen of op tijd te behandelen, nog voor aanpassing aan de gastheeromgeving. Alleen dan is er een redelijke kans de bacterie weg te behandelen met intensieve antibioticatherapie.

Onderzoeksvragen

Het onderzoek beschreven in dit proefschrift gaat in op bacteriële karakteristieken, vroege opsporing en interventie van *P. aeruginosa* infecties bij kinderen met CF.

De volgende onderzoeksvragen worden in dit proefschrift behandeld:

- Wat zijn de karakteristieken van *P. aeruginosa* bacteriën welke een eerste infectie veroorzaken bij kinderen met CF? Zijn de karakteristieken van bacteriën welke een snel een chronische infectie veroorzaken anders dan die van bacteriën welke wegbehandeld kunnen worden?
- Wat is de waarde van de huidige diagnostische technieken om een *P. aeruginosa* luchtweginfectie aan te tonen? Kan de diagnostiek verbeterd worden door het meten van serum anti-*Pseudomonas* antistoffen?

- Kan een eerste infectie met *P. aeruginosa* bij kinderen met CF voorkomen of uitgesteld worden door driemaandelijkse profylactische antibioticabehandeling met ciprofloxacine en colistine?
- Wat zijn de nadelen van het gebruik van profylactische antibiotica bij patiënten met CF?

Resultaten

Bacteriële karakteristieken

De meeste patiënten met CF zullen ooit een infectie met *P. aeruginosa* doormaken. Tegenwoordig kan na tijdige opsporing een eerste infectie bij zo'n 60-90 % van de patiënten succesvol behandeld worden met intensieve antibioticatherapie. Desondanks zijn er patiënten bij wie de behandeling faalt waarna een chronische infectie ontstaat. Om te achterhalen waarom bij sommige patiënten met een eerste infectie de therapie niet succesvol was, werd verder gekeken naar specifieke karakteristieken van *P. aeruginosa* bacteriën van een grote groep patiënten met een eerste infectie. Daarnaast werden *P. aeruginosa* bacteriën van kinderen met CF vergeleken met *P. aeruginosa* bacteriën van kinderen zonder CF maar met een infectie veroorzaakt door *P. aeruginosa*, zoals chronische otitis of een urineweginfectie. Hoofdstuk 2 beschrijft genetische typeringen (genotypen) van 202 *P. aeruginosa* bacteriën. Het bleek dat de genotypen erg divers waren bij kinderen met CF met een eerste infectie en bij kinderen zonder CF. Bij patiënten met CF met een chronische *P. aeruginosa* infectie was de diversiteit veel minder en werd een veelvoorkomend, zogenaamd klonaal genotype geobserveerd bij ongeveer 50 % van deze patiënten. Dit klonale genotype werd ook gevonden bij 2 kinderen met CF en een eerste infectie, maar niet bij de kinderen zonder CF. Deze bevinding suggereert een gezamenlijke oorsprong of overdracht (kruisinfectie) en/of een gastheergevoeligheid van de patiënt met CF voor het specifieke klonale genotype. Bij de 2 kinderen met CF welke een eerste infectie doormaakten met het klonale genotype, was het niet mogelijk de bacterie weg te behandelen ondanks meerdere antibioticabehandelingen. Dit genotype lijkt dus succesvol in het chronisch infecteren van de luchtwegen van patiënten met CF.

Beperkt onderzoek naar dit klonale genotype laat zien dat dit een aangepaste bacterie is welke weinig virulentiefactoren uitscheidt en meestal gevoelig is voor meerdere antibiotica. De verminderde uitscheiding van virulentiefactoren zou een reden kunnen zijn dat deze bacterie niet bij andere dan CF-patiënten voorkomt. Echter de mate van virulentie alleen bepaalt niet het succes van het veroorzaken van een chronische infectie ondanks intensieve antibiotica behandeling. Hoofdstuk 3 beschrijft karakteristieken van eerste *P. aeruginosa* bacteriën van patiënten met CF. Een vergelijking wordt gemaakt tussen de karakteristieken van wegbehandelde bacteriën (het merendeel) en van bacteriën welke een chronische infectie veroorzaakten. Bij *P. aeruginosa* bacteriën welke een eerste infectie veroorzaakten en waarbij intensieve antibioticatherapie niet succesvol was, werd een grote variatie gezien in de virulentie, cytotoxiciteit en de mutatiefrequentie. De cytotoxiciteit was gemiddeld hoger maar het voorspellen van behandelsucces op basis van bacteriële karakteristieken alleen

bleek gezien de grote variatie niet mogelijk. Snelle progressie naar een chronische infectie ondanks intensieve antibiotica therapie zal waarschijnlijk soms worden bepaald door de bacterie (in het geval van het succesvolle klonale genotype) maar waarschijnlijk meestal door de complexe gastheer-pathogeeninteractie.

Vroege opsporing

Het tijdig kunnen opsporen van *P. aeruginosa* is zowel afhankelijk van de manier van afname als microbiologische technieken. Hoofdstuk 4 geeft een samenvatting van de literatuur over diagnostische mogelijkheden. Tot nu toe wordt *P. aeruginosa* meestal geïdentificeerd door het kweken van sputum of een keel-hoestwat op selectieve kweekplaten. Omdat jonge kinderen meestal geen sputum kunnen opgeven, wordt als alternatief een keel-hoestwat afgenomen voor kweek. De diagnostische waarden van een keel-hoestwatkweek voor aanwezigheid van *P. aeruginosa* in de longen zijn echter lager. Dit zou kunnen leiden tot het later ontdekken van *P. aeruginosa* in de luchtwegen. Naarmate keel-hoestwatten vaker worden afgenomen, stijgt de voorspellende waarde voor aan- of afwezigheid van *P. aeruginosa* in de longen. Bij een herhaalde positieve kweek is de kans dus groter dat *P. aeruginosa* zich in de longen bevindt. Gegevens uit de prospectieve studie (hoofdstuk 6) laten echter zien dat de meeste positief bevonden keel-hoestwatkweken werden gevolgd door een opnieuw positieve kweek. Dit rechtvaardigt het geven van een antibioticabehandeling na een enkele positieve keel-hoestwat. Het faciliteren van het opgeven van sputum door middel van het vernevelen met een hypertone zoutoplossing leidt niet tot het significant extra detecteren van *P. aeruginosa*, daarom wordt routinematige invoering van deze techniek niet aanbevolen. Naast de traditionele kweek zijn er nieuwere moleculaire technieken zoals de polymerase kettingreactie (PCR) welke een snellere detectie van *P. aeruginosa* mogelijk maken, waarbij er tevens de mogelijkheid bestaat om specifieke genotypen en kruisinfecties op te sporen.

De bijdrage van het meten van anti-*Pseudomonas* antistoffen in het bloed om een eerste *P. aeruginosa* infectie te diagnosticeren is uitgebreid bestudeerd maar nog niet conclusief (hoofdstuk 4). Hoofdstuk 5 beschrijft de waarde van serum antistoftesten met 3 specifieke *P. aeruginosa* antigenen (in deze studie 3 verschillende virulentiefactoren). Antistoftesten gebaseerd op virulentiefactoren hangen af van de expressie van die factoren. De betreffende testen hebben een hoge diagnostische waarde in het aantonen van chronische infectie, echter slechts rond een derde van de patiënten met een eerste infectie hebben serum antistoffen ten tijde of alvorens de directe detectie van *P. aeruginosa* in een kweek. In de prospectieve studie beschreven in hoofdstuk 6 lag dit getal nog lager. Daarom hebben deze specifieke antistoftesten als screenend instrument bij een grote groep CF-patiënten weinig aanvullende waarde. Antistoftesten met andere antigenen hebben mogelijk betere resultaten. Het is echter de vraag of er bij aanwezigheid van alleen anti-*Pseudomonas* antistoffen in het bloed zonder een positieve kweek ook antibiotische behandeling ingezet moet worden. De aanwezigheid van antistoffen kan tijdelijk zijn, zoals beschreven in betreffende hoofdstukken. Daarnaast is het nog niet duidelijk of patiënten met een chronische infectie voordeel zouden hebben gehad bij een eerdere detectie in het vroege infectiestadium door middel van antistoftesten en daaropvolgende behandeling.

Interventie

Om te bepalen of een eerste *P. aeruginosa* infectie bij kinderen met CF voorkomen kan worden, werd een prospectieve studie uitgevoerd. Hoofdstuk 6 beschrijft een gerandomiseerde placebo-gecontroleerde trial met 65 deelnemers in de leeftijd van 0 tot 18 jaar. De deelnemers waren allen kinderen met CF zonder aanwijzingen voor een infectie met *P. aeruginosa* (kweek en antistoffen) in de 2 jaar voorafgaand aan de trial. Vierendertig kinderen kregen om de 3 maanden een 3-weken durende behandeling met twee antibiotica, te weten ciprofloxacine per os en colistine inhalatie. De 31 andere kinderen kregen hetzelfde behandelschema, echter met twee identieke placebo's. Patiënten, onderzoekers en behandelaars waren geblindeerd voor de behandeling. Na de 3-jarige onderzoeksperiode waren er 19 kinderen met een eerste *P. aeruginosa* infectie, 10 in de placebo groep en 9 in de antibioticagroep. Er was geen statistisch significant verschil in het krijgen van eerste en chronische *P. aeruginosa* infectie tussen de 2 groepen. Na 3 jaar was de afname in longfunctie voor beide groepen gelijk. Het aantal kinderen met anti-*Pseudomonas* antistoffen was hoger in de placebogroep in het 1e jaar van de studie, maar er waren geen verschillen meer na 3 studiejaren. Het antibioticumgebruik naast de trialbehandeling ten behoeve van luchtwegklachten was niet significant verschillend tussen de twee groepen. Een belangrijke bevinding in deze trial was dat profylactische antibioticabehandeling leidde tot een verandering in bacteriële kolonisatie van de luchtwegen. Er werden onder andere vaker gram-negatieve niet-fermenterende bacteriën gevonden in de antibioticagroep. Deze bacteriën zijn vaak intrinsiek resistent voor antibiotica. Overigens werd geen ciprofloxacine of colistine resistentie waargenomen bij *P. aeruginosa* gekweekt bij patiënten uit de antibioticagroep. Waarschijnlijk is het cyclische profylaxeschema onvoldoende intensief om *P. aeruginosa* infecties bij de meeste kinderen met CF te voorkomen. Intensiveren van het profylaxeschema zal allicht meer verschuiving van bacteriële kolonisatie in de hand werken.

Onderhoudsbehandelingen met macrolide antibiotica zoals azithromycine worden voorgeschreven voor CF-patiënten met een chronische *P. aeruginosa* infectie. Het werkingsmechanisme van dit antibioticum is niet zozeer gerelateerd aan een direct antibiotisch effect; in-vitro onderzoek toont aan dat macrolide antibiotica interfereren met het bacteriële systeem wat virulentiefactoren produceert, het quorum-sensing systeem. Daarnaast hebben zij ontstekingsremmende eigenschappen. Hoofdstuk 7 beschrijft het beloop van de longfunctie na het initiëren van azithromycine onderhoudsbehandeling. In het eerste jaar werd een verbetering van longfunctie gezien; de jaren daarna daalde de longfunctie echter weer. Binnen een jaar ontstond bij 80 % van de patienten welke gekoloniseerd waren met *S. aureus* resistentie van *S. aureus* voor macrolide antibiotica. Het ontwikkelen van macrolide resistentie was niet gerelateerd aan het longfunctiebeloop; dit beloop was voor de groepen met en zonder macrolide resistente *S. aureus* gelijk.

Omdat ongeveer 30 % van alle mensen *S. aureus* in de neus draagt, en er transmissie van *S. aureus* binnen gezinnen is beschreven, werd gekeken of er overdracht was van macrolide resistente *S. aureus* tussen CF-patiënten en hun gezinsleden. Dit kan van belang zijn omdat het dragen van *S. aureus* een risicofactor vormt voor het ontwikkelen van infecties met *S. aureus*. Omdat macrolide resistentie vaak gepaard gaat met clindamycine resistentie, zijn in

dat geval de therapeutische opties verminderd. Hoofdstuk 8 beschrijft *S. aureus* dragerschap en het resistentiepatroon bij 194 gezinsleden van 65 CF-patiënten. Door middel van genotyperingen en resistentiebepalingen van *S. aureus* werd aangetoond dat bij gezinsleden van CF-patiënten niet vaker een macrolide resistente *S. aureus* werd gevonden. Individueel antibioticagebruik was meer bepalend voor het dragen van een macrolide resistente *S. aureus* dan het hebben van een gezinslid met CF en gekoloniseerd met een macrolide resistente *S. aureus*.

Conclusies

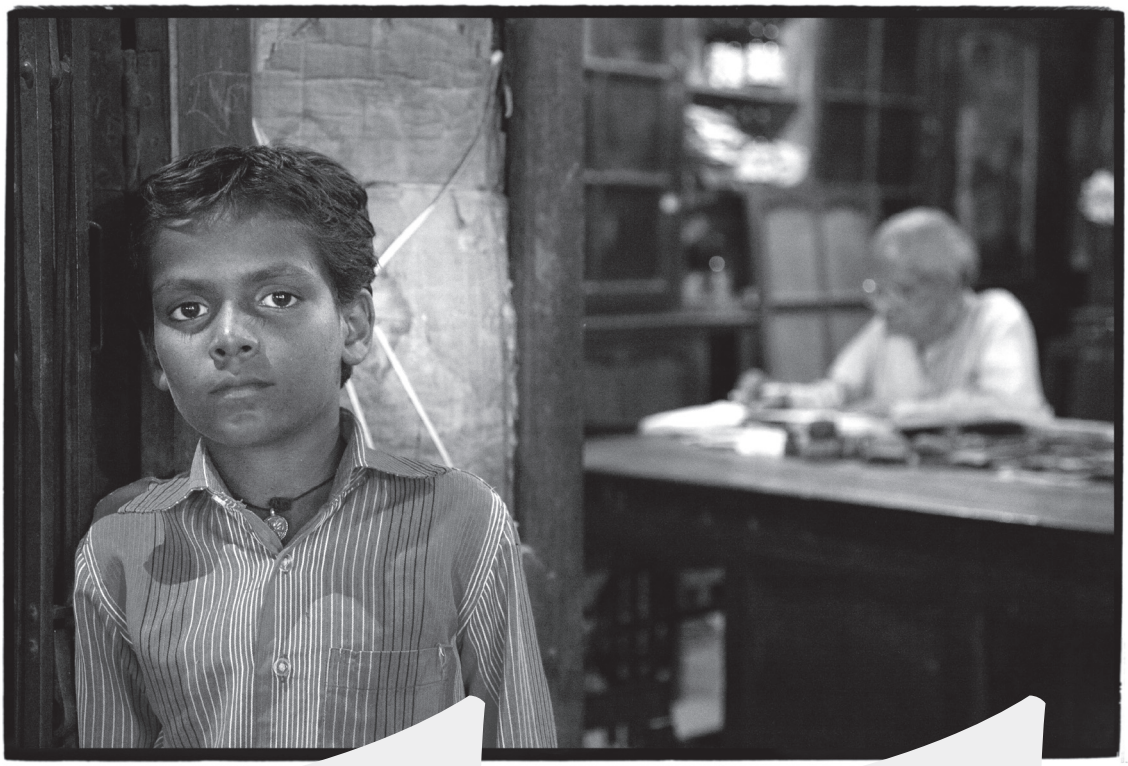
De belangrijkste conclusies van dit proefschrift zijn:

- *P. aeruginosa* bacteriën van kinderen met CF worden gekenmerkt door een lagere genetische diversiteit dan *P. aeruginosa* bacteriën van kinderen zonder CF. Een specifiek klonaal genotype werd alleen bij kinderen met CF geobserveerd.
- Bacteriële karakteristieken van *P. aeruginosa* van een eerste infectie bij CF-patiënten zijn erg variabel. Het is niet mogelijk om op basis van alleen deze bacteriële karakteristieken te voorspellen welke CF patiënt een groter risico heeft op een chronische infectie ondanks intensieve antibiotica behandeling.
- Het regelmatig afnemen van een keel-hoestwat voor kweek geeft een goede voorspellende waarde voor aanwezigheid van *P. aeruginosa* in de longen.
- Specifieke serum antistofbepalingen dragen niet substantieel bij aan vroege opsporing van eerste *P. aeruginosa* infectie bij de meeste kinderen met CF maar hebben wel een hoge diagnostische waarde voor een chronische infectie.
- Cyclische 3-maandelijkse anti-*Pseudomonas* profylaxe is onvoldoende intensief om een eerste *P. aeruginosa* infectie en bijkomende longfunctieachteruitgang te voorkomen. Een verandering in bacteriële kolonisatie werd geobserveerd bij kinderen welke 3-maandelijkse profylaxe kregen.
- Onderhoudsbehandeling met macrolide antibiotica geeft een tijdelijk longfunctieherstel maar leidt tot snelle macrolide resistentie bij *S. aureus*. Significante transmissie van macrolide-resistente *S. aureus* naar gezinsleden van CF-patiënten werd niet aangetoond.

Toekomst

Met betrekking tot bacteriële factoren is het belangrijk de reden van het succes van infectie van CF-patiënten door klonale genotypen te bestuderen. Dit kan mogelijk ook suggesties geven voor de reden van het falen van een intensieve antibioticabehandeling bij een eerste infectie met niet-klonale genotypen. Daarnaast is er nog weinig bekend over co-infectie met andere pathogenen, zoals virussen, en het effect op *P. aeruginosa*. Interacties tussen pathogenen zijn waarschijnlijk van belang in het gehele infectieproces, zowel met betrekking tot een eerste infectie als het opvlammen van een chronische infectie. Om een *P. aeruginosa*

infectie tijdig op te kunnen sporen, zijn nieuwe sensitievere en snellere opsporingstechnieken nodig. Een voorbeeld is een multiplex PCR assay. Hierbij kunnen ook andere mogelijk relevante pathogenen worden meegenomen om meer te weten te komen over de interactie en dynamiek van het infectieproces. Daarnaast zijn gastheerfactoren in de aangeboren immuniteit tegen *P. aeruginosa* nog onopgehelderd. Het focus met betrekking tot profylaxe moeten liggen op die patiënten met een extra risicovol profiel, bijvoorbeeld op basis van verschillen in het aangeboren immuunsysteem en situaties welke een grotere kans geven op infectie met *P. aeruginosa*, zoals voorafgaande (virale) infecties. Voor behandeling van een eerste *P. aeruginosa* infectie moet het effect van adjuvante therapie naast antibiotische behandeling prospectief getest worden. Voorbeelden van adjuvante therapie zijn quorum-sensingremmers zoals azithromycine of anti-oxidanten.



Boy in front of traditional Ayurveda doctor - Varanasi, India

About

Who is...?
List of publications
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Who is...?

Gerdien Tramper was born May 17, 1977 in Yerseke, the Netherlands and is married to Jonathan Stranders.

Professional education

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Research projects

2004 - 2009 PhD research project 'Respiratory infections with *Pseudomonas aeruginosa* in children with cystic fibrosis **| 2006 - 2009** Research collaboration project with Danish Technical University and Rigshospitalet, Copenhagen Denmark **| 2006** ESPID small grant award **| 2001** Scientific internship 'Tuberculosis among children in Sudan: an investigation into factors that delay diagnosis' - National Tuberculosis Programme, Khartoum Sudan

Personal interests

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Chapter 2

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Chapter 5

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