Bacterial colonization of the respiratory tract in patients with cystic fibrosis

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Bacterial colonization of the respiratory tract in patients with cystic fibrosis

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in patiënten met taaislijmziekte

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Chapter 1

Introduction

CYSTIC FIBROSIS: GENERAL FEATURES

Cystic fibrosis (CF) is the most common single gene disorder in The Netherlands and occurs approximately once in every 3600 children born alive (1). The heterozygous carrier frequency has been estimated to be approximately 1:30.

The defective gene was identified in 1989 and appeared to be located on chromosome 7. It codes for the cystic fibrosis transmembrane conductance regulator (CFTR), which acts as a transmembrane chloride channel. The most frequent mutation of this gene is the deletion of phenylalanine at position 508 (ΔF508). Almost 60% of the known CF patients in The Netherlands are homozygous for the ΔF508 mutation (2). More than 700 additional CFTR mutations related to CF have been identified as of late May 1997 by the CF Genetic Analysis Consortium (3).

The gene defect results in significant morbidity and affects mainly the respiratory tract and the pancreas. The CF lung presents an unique environment to microbial pathogens. The combination of low or absent chloride secretion and an increased sodium absorption results in relative dehydration of the airways. Consequently, the disease is characterized by the production of abnormally viscid secretions in epithelial tissues. Mucociliary clearance of bacteria from the lungs is impaired because of the viscid, dehydrated nature of the airway epithelia. Chronic airway inflammation leads to excessive secretion of purulent mucus and to obstruction of the airway which in turn causes bronchiectasis, pulmonary hypertension with cor pulmonale, haemoptysis, pneumothorax and, finally, respiratory failure. The exacerbation of pulmonary infections is the major cause of morbidity and mortality in patients with CF. Aggressive early treatment of respiratory infections is a critical success factor in the treatment of CF patients. Thirty years ago, most patients died in infancy. Nowadays, patients born in the 1990's are likely to live up to a median age of 40 years (4).

COLONIZATION OF THE RESPIRATORY TRACT

Ordinarily, CF patients are colonized by and sometimes infected with a variety of potential microbial pathogens, almost any of which can cause serious lung infections. The respiratory pathogens most commonly isolated include *Staphylococcus aureus*, non-encapsulated *Haemophilus influenzae* and *Pseudomonas aeruginosa*. *S. aureus* and non-encapsulated *H. influenzae* are isolated early in life. Later in life, nearly all CF patients become infected with *P. aeruginosa*. In a recent study 81% of all CF lung infections in adults appeared to caused by *P. aeruginosa*, with only 30% attributable to *S. aureus* and 8% to *H. influenzae*, the most common childhood infective pathogens (5). The species *P. aeruginosa* is known for its extraordinary metabolic and genetic versatility resulting in the production of multiple virulence factors. The species can become very easily resistant to many commonly used antibiotics. Eradication of *P. aeruginosa* from the respiratory tract of CF patient is virtually impossible, and many patients harbor the organisms as their dominant respiratory pathogen for many years. A major factor which is responsible for the long-term persistence of *P. aeruginosa* in the lung is the bacterium's switch to the mucoid phenotype (6). Most patients are colonized with a non-mucoid phenotype in the beginning. The timing and causes of the mucoid switch are presently unknown. When the *P. aeruginosa* strain produces an alginate capsule or mucoid exopolysaccharide (MEP) and thus attains a mucoid phenotype, this is associated with a progressive decline in pulmonary function of the CF patients (6). CF patients who harbor non-mucoid *P. aeruginosa* and / or harbor only *S. aureus* maintain over 90% of their expected lung function for many years. The presence of *S. aureus* in the absence of mucoid *P. aeruginosa* at the age of 18 correlates with long-term survival of CF patients (7). The presence of slow-growing subpopulations of *S. aureus* and *P. aeruginosa* (termed small colony variants or SCVs) has recently been associated with CF (8, 9). The SCV *S. aureus* and *P. aeruginosa* can be cultured after exposure in vivo to aminoglycosides and exhibit decreased susceptibility against antimicrobial agents.

Other bacterial pathogens cultured from the CF respiratory tract are generally not persistent colonizers; these intermittent species include *Streptococcus pneumoniae*, *Escherichia coli*, *Klebsiella spp*, *Proteus spp*, *Serratia spp*, *Enterobacter spp*, *Citrobacter spp* and group A *streptococci* (10). Many other species belonging to the group of gram-negative non-fermenters

have been isolated from the respiratory tract of CF patients, including *Pseudomonas spp*, *Acinetobacter anitratus*, *Achromobacter spp*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia* ((10). Less often and later on in life, CF patients can become infected with organisms like *Aspergillus spp*, *Candida albicans* and atypical mycobacteria. Colonization of the previously mentioned microorganisms is intercurrent and is not associated with apparent clinical deterioration, with the exception of *B. cepacia*. The clinical course of patients following *B. cepacia* colonization and infection is variable. Of particular concern is a subset of patients who experience an acute syndrome, usually ending in death, characterized by severe and unexpected pulmonary complications (6). The prevalence of *B. cepacia* in The Netherlands is estimated to be less than 3% (11).

DETECTION OF MICROORGANISMS IN THE RESPIRATORY TRACT OF CF PATIENTS

In the clinical situation sputum of CF patients produced during coughing is routinely cultured. Washed sputum specimens should be plated onto several media including Columbia agar containing 5% sheep blood, MacConkey agar and chocolate agar. Additional selective media are used to isolate a specific microorganism despite the presence of large numbers of bacterial cells belonging to other species, for instance *P. aeruginosa*. Mannitol salt agar is a selective medium for *S. aureus* and it is used because the growth and detection of *S. aureus* may otherwise be hampered by the presence of mucoid *P. aeruginosa* (12). Thymidine-dependent *S. aureus*, which may appear during treatment with trimethoprim-sulfamethoxazole, are also able to grow on mannitol salt agar. *S. aureus* small colony variant (SCV) grow as non-hemolytic, non-pigmented and very small colonies on these media (< 1mm)(8). *P. aeruginosa* SCV are able to grow on 5% Columbia agar and MacConkey agar plates. SCV morphotypes of *P. aeruginosa* vary in colony size between 1-3 mm after 48 hours of incubation (9). Enriched chocolate agar with bacitracin is recommended to culture *H. influenzae* (13). For specific isolation of *B. cepacia* a selective medium containing polymyxin B and bacitracin (OFPBL) or a medium containing ticarcillin and polymyxin B (PC agar) is recommended (14,15).

For the broad spectrum molecular detection of microorganisms from sputa from CF patients, 16S rRNA has been used as target molecule. rRNA molecules contain several functionally different regions, some of which have conserved sequences and others which are highly variable. The 16S rRNA sequence of a species is a stable genotypic feature which may be useful for the identification of microbes at the genus or species level. DNA probes based on the sequences of these unique target regions are useful in identifying bacterial species. Recent studies have employed this technique to identify *H. influenzae*, *S. aureus* and *P. aeruginosa* as a pathogen in various infections (respectively 16-18, 19-21, 22, 23). The molecular technique targeted in our study was bacterial genes for the small subunit ribosomal RNA. These genes were amplified from DNA extracted from CF sputum samples, cloned and characterized by hybridization and DNA sequencing (24). Oligonucleotide hybridization has performed with probes for the three major CF pathogens (25).

METHODS OF STRAIN TYPING

The focus of this part of the introduction is the process of analyzing multiple isolates within a given species to determine whether they represent a single strain or multiple different strains. The detailed analysis of multiple isolates can contribute to the development of new insights into both the epidemiology and the pathogenesis of infection. The epidemiology of colonization of CF patients has been studied extensively. First, phenotyping techniques were used but around the beginning of the '90-ies the genotyping techniques were introduced. The ability to differentiate strains of *P. aeruginosa* and *S. aureus* from medical and environmental sources and to track patient-to-patient and/or intrafamilair exchange of strains, depends on the use and interpretation of available typing methods. Over the years, several typing methods have become accepted as means of strain identification. However, each method has its limitations, especially if they are used alone, are not carefully standardized, and, if samples are not tested repeatedly.

Only a few pheno- and genotyping techniques have been used regularly for typing of colonizing microorganism from CF patients, and these have been applied in our studies. The phenotyping techniques, serotyping and pyocin typing, were used for analyzing of

P. aeruginosa. For typing of *S. aureus* we did not use a phenotyping technique because it was already determinated that molecular methods of typing are superior to phenotyping techniques for *S. aureus* (26). For our study of *H. influenzae* we used major membrane protein profile (MOMP) as phenotyping technique.

CRITERIA FOR EVALUATING TYPING SYSTEMS

The results of an individual typing technique must be considered in relation to the available epidemiological data or to the results of other techniques. Several criteria are useful in evaluating typing techniques: typeability, reproducibility, discriminatory power, ease of interpretation and feasibility (26). Typeability refers to the ability to obtain a result for each analyzed isolate; nontypeable isolates are those that give either no results or uninterpretable results. Reproducibility refers to the ability of a typing technique to yield the same result when the isolate is tested repeatedly or in different institutions. Discriminatory power refers to the ability to differentiate among unrelated isolates. Ideally, each unrelated isolate is detected as unique. The ease of interpretation for phenotyping techniques is less complicated then for genotyping techniques. The interpretation of data obtained by phenotyping techniques is based on the presence of absence of metabolic or biologic activities as expressed by the whole organisms. The results of genotyping techniques show typically patterns of "bands", each band representing a discrete bacterial product or DNA fragment. Such patterns may be extremely complex, and difficult to analyze by using logical, objective criteria. A typing technique is feasible when it is inexpensive and technically accessible, produces rapidly available results, requires neither special expertise, expensive equipment, nor restricted reagents and is useful for a broad range of microorganisms.

PHENOTYPING TECHNIQUES

1. Serotyping

Strain differentiation for *P. aeruginosa* by serological techniques is based on the diversity in a heat-stable, somatic antigen group -the O antigens- and is generally considered the most stable and easily applied system. In an effort to standardize the serological typing system, an International Antigenic Typing Scheme has been proposed, which defined 17 standard antigenic strains (27). Commercially prepared antisera from these strains are available. However, serotyping is limited by it's relatively poor level of typeability and discriminatory power. Because certain serotypes occur with high frequency, the serotype system may not be discriminatory if many or even all strains under investigation possess the same O antigen.

2. Pyocin typing

Pyocins are antibacterial substances (bacteriocins) that are produced by *P. aeruginosa*. *P. aeruginosa* can produce several pyocins and because the production of pyocins varies considerably among strains, the pattern of pyocin production has been successfully employed as the basis for a typing scheme. Pyocin production involves measuring a test organism's sensitivity to a set of standard pyocins. Standard pyocin indicator strains and methodology are available (28). One of the major difficulties of pyocin typing is the instability of a pyocin pattern, since a given pyocin pattern may change with bacterial metabolism. While growth conditions of the test organism can be standardized in the laboratory, non-bacterial factors such as previous antibiotic therapy may alter pyocin productions patterns, leading to false conclusions regarding the rate of colonization by new strains. Pyocin typing is useful, however, when applied in conjunction with serological typing in order to further differentiate organisms belonging to the same O-antigen group.

3. Major Outer Membrane Protein profiling (MOMP)

For MOMP profiling, cell envelops have to be isolated. The protein composition of these envelops can be assessed using denaturing polyacrylamide gelelectrophoresis (SDS-PAGE) (29). Subsequent Coommassie Brilliant Blue staining reveals the presence of different variants of a limited number of MOMP's. Using monoclonal antibodies, the individual MOMP's can

be further recognized and the differences in molecular weight of similar proteins from different strains can be determined (30). The profile, whether or not completed with Western blotting, provides a distinct, strains specific characteristic that can be used for (epidemiological) typing purposes.

A phenotypic conversion of *P. aeruginosa* is characteristic for the CF lung habitat. Strains vary in the composition of peripheral sugar chains of lipopolysaccharide (LPS), their repertoire of bacteriophage receptors and the production of pyocins. In conclusion, although phenotyping techniques have been of value, such techniques have limited typeability and discriminatory power. Because phenotypic conversions of *P. aeruginosa* are characteristic for strains in the CF lung habitat, these techniques are further hampered by problems in stability and thus reproducibility. Therefore phenotypic techniques have largely been replaced by modern genotyping for the study of the epidemiology of *P. aeruginosa*.

GENOTYPING TECHNIQUES

All genotyping techniques are based on the fact that bacterial strains belonging to the same species, i.e. isolates of a given species, are likely to more or less differ from each other at the level of their DNA sequence. Several commonly used genotypic techniques are:

1. Random amplification of polymorphic DNA (RAPD) or arbitrarily primed PCR (AP-PCR).

This method is based on the differential amplification of DNA fragments using the polymerase chain reaction (PCR). The method is unique in that it uses random primers that bind differently to the individual genome molecules, which has a clear effect on amplification profiles (31). Genomic DNA is amplified under relatively nonstringent annealing conditions and with only a single primer of arbitrary sequence to initiate amplification. Following electrophoretic separation of amplified fragments, banding pattern comparison is used to classify isolates into related and unrelated groups (Figure 1). The DNA fingerprint is characteristic for a strain.

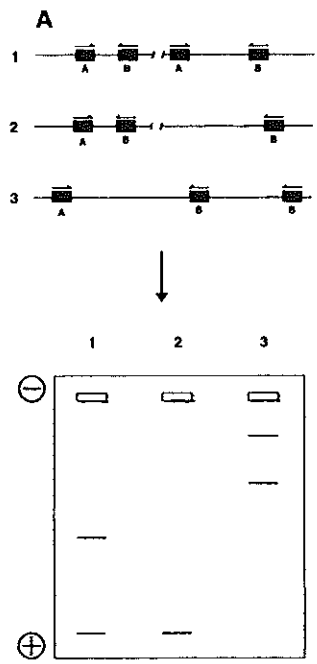

Figure 1 PCR-mediated DNA fingerprinting: primer-binding site variation. DNAs 2 and 3 lack a site present in DNA 1. This results in disappearance of a band in the electropherogram. In this example, multiple primers included in a single PCR may enhance the number of polymorphic sites that can be detected.
(Source Van Belkum A, Clin Microbiol Rev 1994;7:176. With permission)

2. Pulsed-field gel electrophoresis (PFGE)

This method characterizes native, non-amplified DNA by digesting of genomic DNA by low-frequency restriction enzymes that cleave the chromosome infrequently, resulting in a relatively small number of larger DNA fragments of 10 to 800 kbp in length (32). These fragments are then separated by gel electrophoresis. Periodic change in the orientation of the electric field during gel electrophoresis allows separation and size determination of these macro restriction fragments. This technique thus relies on the variation in the electrophoretic mobilities of large restriction fragments of DNA and on the number restriction sites and sizes of the fragments.

3. Variable Number of Tandem Repeats (VNTR)

Repetitive DNA consists of multimeric tracts of certain nucleotide motifs (33). These can be one nucleotide (homopolymeric) in length up to much longer units. Variable number of tandem repeat (VNTR) loci consist of repeat units present in a single locus and showing inter-individual length variability. With DNA primers bordering constant sequence elements up- or down stream of the VNTR, polymorphism in the repeat unit number is documented after DNA amplification. Regions bordering the repeats are generally sufficiently well-conserved targets to allow for PCR-mediated amplification (Figure 2). Basically, at the genome level the variability arises during replication. Because repeat units are identical, neighboring pairs of repeat units can form erroneous hybrids. These structures cause the DNA polymerase to slip (slipped strand mispairing (ssm) see Figure 2), which leads to incorrect replication: repeat units are added or deleted. This variability can serve as a molecular clock and, consequently, provides DNA type information.

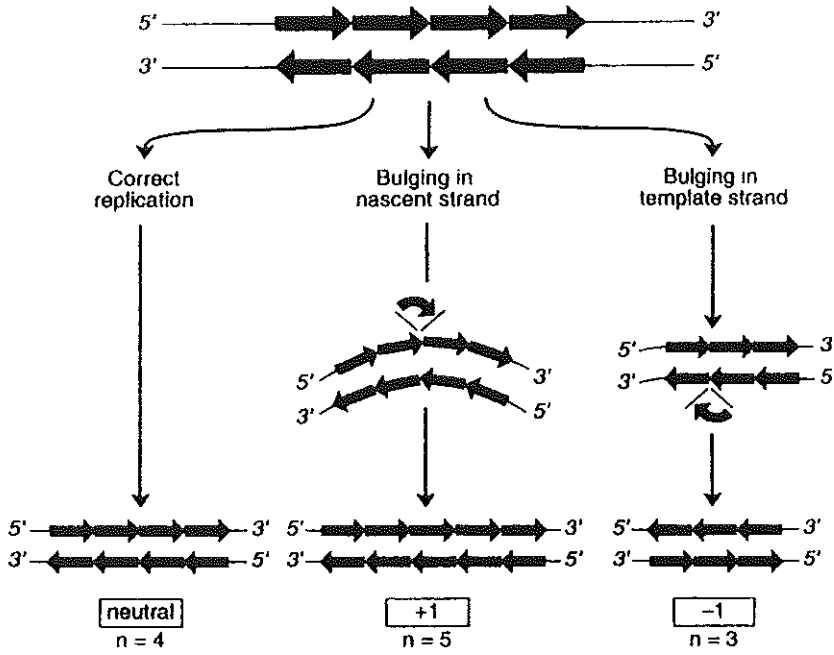

Figure 2: Schematic representation of the mechanism of SSM during replication, which results in shortening or lengthening of SSRs. Individual repeat units are identified by arrows; bulging is the presence of non-base-pair base residues interrupting a regular 2-strand DNA helix. Bulging in the nascent strand leads to a larger number of tandem repeat units; bulging in the template strand results in a smaller numbers of units. During replication, bulges can occur in both strands, and the effect of insertion or deletion can be neutralized by occurrence of the adverse event. The number of repeat units can decrease or increase by multiple repeats once multiple bulging in one strand has occurred.
(Source Van Belkum A, Microbiol Molecul Biol Rev 1998:62:277. With permission).

EPIDEMIOLOGY OF RESPIRATORY TRACT INFECTIONS

The lungs of a CF patient are often colonized with various bacteria and airway inflammation is already documented at an early age. The main pathogens isolated from the lower respiratory tract of these patients are *S. aureus*, *H. influenzae* and *P. aeruginosa*. Epidemiological studies using pheno- and / or genotyping techniques have not been able to resolve the confusion about the routes of transmission, the prevalence of cross-infection, the eventual prevalence of a predominant CF 'type', and the number of strains which may colonize an individual patient. Several typing techniques have been used to gain insight in the epidemiological questions concerning bacterial infections in CF patients.

Staphylococcus aureus

The bronchial colonization of a CF patient is initiated during infancy, most commonly, by a strain of *S. aureus* (5). However, the epidemiology of *S. aureus* in CF patients has not been extensively studied. It has not been clearly demonstrated whether the same strain or different strains are responsible for repeated broncho pulmonary infections or colonization. It is not known wether there are certain types or clones of *S. aureus* that are preferentially associated with cystic fibrosis.

In 1981, phage typing was performed by Shapera et al, on initial and selected follow-up *S. aureus* isolates from CF patients exposed to repeated courses of antistaphylococcal therapy (34). The original *S. aureus* phage type was supposedly by eradicated from the sputum of 12 out of 29 patients. However, during the follow-up period (3-6 months) *S. aureus* colonization recurred in 10 of these 12 patients. The same phage type was detected again in seven patients and a new phage type was found in three patients. Earlier studies had shown no significant differences in the distribution of phage types among *S. aureus* isolates from CF and non-CF patients. Thus, no predominant phage type appeared to be associated with the CF population (35, 36, 37). Albus et al investigated the *S. aureus* capsular types in CF patients and healthy individuals (38). Capsular type 8 strain predominated with a relatively small number of strains with capsular type 5 and a similar percentage of strains that were non-typeable. However, also these capsular types seemed to be randomly distributed between the two groups.

An epidemiological analysis of *S. aureus* from 34 CF patients cultured over a period of 30

months was studied by bacteriophage typing, plasmid profiling, and (in some instances) chromosomal restriction fragment pattern analysis (39). Only six out of thirty-four patients appeared to be persistently colonized by one type (for up to 10 months) on the basis of plasmid and phage typing. Identical types were observed in 68% of the patients with as many as five individuals sharing the same identical strain type. In a French study, *S. aureus* isolates were tested against 14 antibiotics and characterized by esterase electrophoretic typing, capsular polysaccharide serotyping and phage typing (40). Again, no *S. aureus* type was specific to cystic fibrosis, the isolates belonging to a wide range of types. Esterase electrophoretic typing indicated that *S. aureus* persisted for long periods in 73% of the patients (followed for at least 6 months). Only three out of eighty-five CF patients had two persistent strains at the same time.

There are no studies about transmission of *S. aureus* and the percentage of cystic fibrosis patients that carry *S. aureus* in the anterior nares is also unknown. However, chronic sinusitis is a common problem in cystic fibrosis patients. The microorganisms responsible for this infection generally are *S. aureus* or *H. influenzae*. Umetsu et al suggested that sinus disease is associated with pulmonary exacerbation in CF patients (41).

Phenotyping and genotyping techniques have demonstrated that identical *S. aureus* isolates can be found in related and unrelated CF patients(39). This could imply that cross-infection exist. However, no evidence has been brought forward that suggest the existence of specific CF *S. aureus* types or clones.

Haemophilus influenzae

H. influenzae, cultured from respiratory secretions of CF patients, are nearly always non-encapsulated (42). Non-encapsulated *H. influenzae* is a commensal of the upper respiratory tract and is found in 50-80% of healthy individuals (43). Occasionally, encapsulated serotype b strains may be recovered, most often from CF children less than five years of age. Some investigators have found biotype 1 to predominate in CF patients (42, 44). Biotype 1 has also been associated with pulmonary exacerbations in CF patients (45). Möller et al analyzed *H. influenzae* strains cultured during a 24 months period from CF patients, by MOMP profiling and RAPD analysis (46). The results showed that all CF patients were infected with different *H. influenzae* strains. Furthermore, CF patients were often colonized by multiple *H. influenzae*

strains, of which some persisted for up to 2 years. The study of Bilton et al showed that large numbers of non-typeable *H. influenzae* were present in sputum from adult CF patients (47). Several biotypes and MOMP types were observed, with no apparent association between these two phenotypic characteristics. Two studies showed that pairs of siblings shared identical genotypes of *H. influenzae* which can indicate that cross-infection does occur (44, 46).

Pseudomonas aeruginosa

With the advent of effective antistaphylococcal therapy, *P. aeruginosa* emerged as the most important bacterial pathogen in lung disease of CF patients (48). A unique feature of CF patients chronically colonized with *P. aeruginosa* is the recovery of an unusual morphotype from respiratory secretions. The first description of this association was made by Doggett et al in the 1960s and since that time, this has been confirmed by others repeatedly (49, 50, 51). The morphotype was designated mucoid and this was due to the production of large amounts of polysaccharide that surround the cell. The material has been designated by Pier as "mucoid exopolysaccharide (MEP) or alginate capsule" (52). A major factor in epidemiological studies of mucoid isolates has been the availability and development of reliable typing methods. In particular techniques that cope with the viscous nature of the bacterial alginate and loss of O-antigenic components of bacterial lipopolysaccharide (LPS) (52). According to the International Antigenic Typing System, which is based on the O-polysaccharide component of lipopolysaccharide, mucoid strains are usually polyagglutinable (53). The polyagglutinability is probably due to a lack of polysaccharide side chains in these lipopolysaccharides (54). The role of phenotypic characteristics for studying the epidemiology of *P. aeruginosa* in colonized CF patients is controversial. Until the late eighties serotype, phage type, pyocin type and antibiogram were used as epidemiological markers. In 1987, Ogle et al developed a molecular probe, cloned from the region for exotoxin A gene (55). The probe appeared to be useful to study the epidemiology of *P. aeruginosa*. The results suggested that genotypes persist during and after antimicrobial therapy and that more than one genotype can be present in the lungs at the same time. In addition, genotypes can also change over time (56). Speert et al used a pilin probe to compare the restriction fragment length polymorphism patterns of sequential isolates collected over a period of seven years from 23 patients (average 4 specimens per patient) and observed that most CF patients are colonized for prolonged period

with a single RFLP type (57). The international *P. aeruginosa* typing study group compared different phenotyping techniques with a genotyping technique for *P. aeruginosa* strains from CF patients (58). The RFLP typing methods seemed to have the best discriminatory power. Since 1990s many studies have used genome macrorestriction analysis to determine diversity and/or variability of *P. aeruginosa* isolates of CF patients (59). The results of macrorestriction of *P. aeruginosa* DNA and genome fingerprinting by PFGE also showed the persistence of single clones in some CF patients (60, 61, 62, 63). Römling et al observed predominant clones in each of four different clinics (60). Recently, genotyping of *P. aeruginosa* from CF patients by arbitrary-primed or random amplified polymorphic DNA PCR has been reported (64, 65, 66). Overall, most CF patients become chronically colonized with a single genotype of *P. aeruginosa* that remains throughout the patient's lifetime (66). However, transient or permanent co-colonization with more than one genotype occurs in 20-30% of all patients. Direct genotyping of *P. aeruginosa* in sputum of chronically colonized CF patients revealed that the airways are colonized with a homogeneous population of the same *P. aeruginosa* genotype (67).

The original source of *P. aeruginosa* responsible for lung infections in CF is still unknown. Possible routes of transmission are endogenous carriage, environmental acquisition, direct patient to patient contact (hospital or elsewhere) or through contamination of the environment by CF sources. Speert et al determined that *P. aeruginosa* isolates of the gastrointestinal tract are not identical to isolates of the lungs (68). Laraya-Cuasay investigated the carrier rates of CF patients and non-CF family members and found no increased carriers (69). None of the CF patients carried *P. aeruginosa* in their nares. Only a few comparative studies of strains isolated from patients and environment were done. In 1983 Zimakoff et al tried to identify the possible reservoirs and routes of cross-infection by taking samples from patients, staff and the environment in a cystic fibrosis centre (70). The study showed that the large reservoir presented by CF patients gives rise to contamination of the environment resulting in cross-infection of other patients. After changing the isolation and hygienic precaution procedures they found no *P. aeruginosa* types shared between environment and CF patients (71). The results of the study of Boßhammer et al showed that rooms reserved for colonized patients were more frequently contaminated with *P. aeruginosa* but no direct exchange between patients' strains and environmental strains was detected (72). However, the frequency of cross-

infection through direct patient to patient contact remains a controversial issue. Although, many studies have tried to establish the incidence of cross-infection (see Table 1).

Burkholderia cepacia

B. cepacia was first described in 1950 by Burkholder as a bacterium responsible for rot of onions (86). In addition, it is commonly found in soil and water. *B. cepacia* has become an important pathogen for CF patients. Acquisition of *B. cepacia* in the lungs of CF patients may be associated with a rapid decline in pulmonary function with increased morbidity and mortality (87). Many epidemiological studies of *B. cepacia*, using different pheno- and genotyping techniques, have reported an identical genotype among CF patients in the same CF centre (88, 89, 90). This suggests that some individuals acquire the organism from other patients, either directly or via the immediately shared environment. Long-term colonization with a single genotype was also documented (91, 92). For this thesis we have not studied *B. cepacia*.

Table 1 A review of literature concerning the occurrence of *P. aeruginosa* cross-infection among cystic fibrosis patients

First Author	Year	CF Center	Summer camp	Cross-infection	Typing technique	ref number
Høiby	1980	+		yes	se, ph	73
Kelly	1982	+		highly unusual	se, py	74
Speert	1982		+	low	se	75
Zimakoff	1983	+		patient↔environment	ph, se	70
Thomassen	1985		+	little evidence	se, asp, ss	76
Pedersen	1986	+		patient-to-patient	se, ph	77
Speert	1987	+		minimal	se	78
Grothues	1988			little evidence	se, ph, py, FIGE	59
Tümmler	1991	+		yes	se, ph, py, PFGE	79
Govan	1992	+		relatively rare	?	80
Boukadida	1993	+		no evidence	FIGE	81
Römling	1994	+		yes	se, ph, py, PFGE	66
Hoogkamp-Korstanje	1994		+	relatively rare	se, ph, py, RAPD	82
Cheng	1996	+		yes	PFGE, fgp	83
Farrell	1997	+		yes	?	84
Adams	1998	+	+	yes	AP-PCR, PFGE	85

Note: phenotyping techniques: se:serotyping, ph: phagetyping, py: pyocin typing, asp: antibiotic susceptibility patterns, ss: serum sentivity assay genotyping techniques: FIGE: field inversion gel electrophoresis, PFGE : pulsed-field gel electrophoresis, RAPD: random amplification of polymorphic DNA, fgp: flagellin gene polymorphisms, AP-PCR: arbitrarily primed polymorphic chain reaction
?: typing method not specified

AIM OF THE STUDIES PRESENTED IN THIS THESIS

Epidemiological studies using phenotyping methods have so far failed to resolve the confusion about the prevalence of cross-infection, and the number of strains which may colonize an individual patient simultaneously and in the course of their disease. Isolates of *P. aeruginosa* with differing morphological characteristics and antibiograms have been cultured from single sputum samples of individual patients. This observation together with reported difficulties in typing of bacterial isolates of CF patients has presented detailed studies into the number of types of a given pathogen species which might colonize an individual CF patient. It is known that microorganisms may be replaced by another species of microorganism or other types belonging to the same microorganism. To gain further insight into these problems, epidemiological investigations based on molecular typing methods are needed.
Therefore, the specific aims of our studies were:

- The evaluation of various typing methods as microbiological tools to help elucidate the epidemiology of respiratory tract infections in patients with cystic fibrosis.
- To study the long-term colonization of microorganisms of the respiratory tract of individual cystic fibrosis patients and their environment.
- To explore molecular methods in the detection and identification of microorganisms in sputum from cystic fibrosis patients.

OUTLINE OF THIS THESIS

In **chapter 2** the discriminatory power of two genotypic and two phenotypic techniques for *P. aeruginosa* is evaluated by analysis of *P. aeruginosa* sputum isolates serially obtained over long intervals from 29 independent CF patients.
In **chapter 3** pulsed-field gel electrophoresis is compared with arbitrary primed polymerase chain reaction for typing *P. aeruginosa* isolates.
In **chapter 4** *P. aeruginosa* isolates recovered from sputum specimens of six pairs of CF siblings during a longitudinal study were analyzed to study the epidemiology, especially the occurrence of cross-infection, by using arbitrary primed polymerase chain reaction as the typing method.
In **chapter 5** *S. aureus* isolates from related and unrelated CF patients were genotypically typed by using arbitrary primed polymerase chain reaction for studying the epidemiology of *S. aureus* infection and the occurrence of cross-infection.
In **chapter 6** the existence of variation in tandem repeat loci in *H. influenzae* isolates during persistent colonization of CF patients is described as is its possible relation to modulation of bacterial virulence.
In **chapter 7** the comparison of microbiological cultivation and molecular methods for detection of *S. aureus*, *H. influenzae* and *P. aeruginosa* in CF sputum samples is described.

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Chapter 2

Typing of *Pseudomonas aeruginosa* strains from patients with cystic fibrosis: phenotyping versus genotyping

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ABSTRACT

Objectives: To assess the discriminatory power of two genotypic and two phenotypic techniques by analysis of *Pseudomonas aeruginosa* sputum isolates obtained with long-term intervals from 29 independent cystic fibrosis (CF) patients.

Methods: Fifty eight strains of *P. aeruginosa* were subjected to serotyping and pyocin production was assessed. Arbitrary primed polymerase chain reaction (AP-PCR) and pulsed field gel electrophoresis (PFGE) were applied in order to detect genetic polymorphisms.

Results: From the results of different typing techniques, it appeared that the numbers of separate types varied between 11 and 43, and the percentage of identical *P. aeruginosa* pairs from individual patients varied between 51% and 72%, depending on the test system used. AP-PCR and PFGE displayed enhanced resolution when compared to serotyping and pyocin typing; both DNA typing techniques generated concordant results, although differences in resolution are apparent. This results in 15% discordance, which may be the result of differences in the definitions of (sub)clonal relatedness as applied for AP-PCR and PFGE, respectively.

Conclusions: Molecular typing techniques are superior to phenotyping where *P. aeruginosa* is concerned. AP-PCR is a fast and useful technique for determining clonality among *P. aeruginosa* strains from chronically colonized CF patients. It is clear, however, that the interpretation of data and comparative analysis of PFGE and AP-PCR results necessitates additional (international) standardization and the development of practical guidelines.

INTRODUCTION

The lungs of patients with cystic fibrosis (CF) provide an ecologic niche which supports the growth of several species of potentially pathogenic microorganisms. *Haemophilus influenzae, Staphylococcus aureus* and *Pseudomonas aeruginosa* are encountered frequently in sputum surveillance cultures of CF patients. *P. aeruginosa* causes significant morbidity and is associated with a reduction in the life-expectancy of individuals suffering from CF (1). For this reason it is important to gain insight in the modes of acquisition and transmission of *Pseudomonas* among patients.

Over time, the lungs of CF patients are consecutively colonized by different populations of microorganisms, indicating complex ecologic dynamics. In order to determine the mechanism enabling bacteria to colonize CF lungs for extended periods of time, it is necessary to perform longitudinal studies for unraveling bacterial population genetics. For determination of the putative clonal origin of the various strains inhabiting the CF patients' lung, several phenotypic and genotypic strategies have been employed. These vary from relatively simple biological assays to complex molecular genetic approaches. Several of these typing procedures have been compared in large, sometimes multi center, typing studies of *P. aeruginosa* and *Burkholderia cepacia* from CF patients (2-5). However, since these studies were performed, several new methods have been brought forward, among which are the arbitrary primed polymerase chain reaction (AP-PCR) (6,7) and pulsed field gel electrophoresis of DNA macrorestriction fragments (PFGE) (8).

The aim of the present study was to compare the widely used phenotyping procedures with the more recent genotypic strategies. For this reason a collection of 58 *P. aeruginosa* strains from 29 cystic fibrosis patients was analyzed by serotyping, pyocin typing, AP-PCR and PFGE.

MATERIALS AND METHODS

All 29 cystic fibrosis patients were recruited in the University Hospital Rotterdam (the Netherlands). The mean age of the patients was 18,6 years (range : 6 to 28) and they had been

admitted to the hospital on up to 10 occasions (median 6) during the study period. Per CF patient, two strains of *P. aeruginosa* were isolated from two sputum samples. The sample was collected during a period of hospitalization or at the time of an out-patient visit. The interval between first and second sample was at least 29 months. Strains were isolated by standard microbiological procedures and the isolates were stored at -70°C. If two or more morphologically different strains were observed upon cultivation, only one was arbitrarily selected and used to assess the discriminative power of the four different typing techniques. The 29 pairs of strains were characterized by serotyping and active pyocin typing as described by Horrevorts (9). The O-antisera that were used were obtained from Diagnostics Pasteur (Marne-la-Coquette, France). AP-PCR was performed as described before (10, 11). Since the efficacy of random primers in an AP-PCR test cannot be predicted accurately, the following primer species were first evaluated using a pilot group of 10 *P. aeruginosa* strains : 1283 (GCGATCCCCA), 1281 (AACGCGCAAC), 1254 (CCGCAGCCAA), 1247 (AAG-AGCCGT), 1026 (GTGGATGCGA), 14307 (GGTTGGGTGAGAATTGCACG), 40730 (GGCCATAGAGTCTTGCAGACAAACTGC), 1290 (TACATTCGAGGACCCCTAAGTG), ERIC 1 (CACTTAGGGGTCCTCGAATGTA) and ERIC 2 (AAGTAAGTGACTGG-GGTGAGCG). The latter two primers were also applied in combination in a single PCR test. Banding patterns were inspected visually, and all different fingerprints, even when only a single different band was observed, were assigned a number. Macrorestriction analysis of DNA by PFGE was done according to the methods described by Kaufmann and Pitt (12). Briefly, bacteria were immobilized in agarose blocks and the DNA was extracted and digested with *Xba*I (Boehringer-Mannheim, Germany). The fragments were separated by PFGE in a CHEF-DR II apparatus (BioRad, Veenendaal, the Netherlands) for 36 h with initial and final pulses of 5 and 25 s, respectively. The gel was stained with ethidium bromide and photographed under UV transillumination. Profiles were compared visually. Identical profiles were considered major PFGE-types and designated by letters. The subclonal variants, i.e. tracks of the same general profile but differing in positions of one up to five bands of the major PFGE-types, are indicated by subnumbers.

RESULTS

First, we assessed the discriminatory power of 10 different AP-PCR primers by studying a limited set of five pairs of *P. aeruginosa*. It appeared that primer 1290 provided maximum discrimination in a single assay (see Figure 1 for some examples). Subsequently, all 58 strains were typed with this particular primer, 1290. All results obtained are summarized in Table 1. Depending on the typing strategy, a certain percentage of strains remained non-typeable. The typeability varies from 100%, 93%, 91% to 66% for AP-PCR, PFGE, serotyping and pyocin typing, respectively. Four strains were non-typeable by PFGE; this was probably related to a high intracellular content of endonucleases (see also reference 13). Apparently, AP-PCR is less sensitive to this type of artifact. The numbers of types that could be identified among the 58 strains by the different techniques were 43 (AP-PCR), 30 (PFGE), 11 (serology) and 14 (pyocin). Based on these data, AP-PCR and PFGE seem to be superior for typing of *P. aeruginosa* isolates.

The different typing techniques (AP-PCR, PFGE, serology and pyocin typing, respectively) scored 51%, 58%, 72% and 66% of the paired isolates as identical. This is in agreement with the decrease in resolution as described above. When the result of the two genotypic analyzes were compared, concordant conclusions with respect to strain identity in pairs from a single CF patient were obtained in 85% of the cases. For serology and pyocin typing, the percentage of concordance was only 58%. When genotypic and phenotypic results were compared, even lower percentages of homology were found. The second strain from patients 3, 6, 8 and 19 and both strains from patients 15 and 28 were identical with PFGE (e type), but were not clustered on the basis of AP-PCR, serotyping and pyocin typing. Strain 1 from patient 8 and both strains from patient 16 were identical with PFGE and different with AP-PCR, serotyping and pyocin typing. Four pairs had a different DNA pattern with AP-PCR and had a subclonal (nearly identical) variant with PFGE.

Table 1 Colonization period and typing results of pairs of *Pseudomonas aeruginosa* strains isolated from 29 chronically colonized CF patients.

			Date isolation		Serotype		Pyocin		AP-PCR		PFGE	
	Gender		Strain	Strain	Strain	Strain	Strain	Strain	Strain	Strain	Strain	Strain
No	(M/F)	Year of birth	1	2	1	2	1	2	1	2	1	2
1	F	1972	5/84	9/90	13	13	35b	35b	1	1	c	c1
2	F	1973	9/85	10/90	6	6	45c	45c	2	2	d	d
3	F	1970	10/85	7/91	14	14	84b	84b	3	3	NT	e
4	M	1964	5/84	3/91	13	16	NT	NT	4	5	j	k
5	F	1966	9/83	5/91	3	3	74c	74c	6	6	a	a1
6	F	1972	11/86	8/91	8	8	74b	74b	7	8	f	e
7	M	1973	3/84	1/91	6	6	41d	NT	9	10	i	NT
8	F	1970	11/84	7/91	NT	NT	NT	NT	11	12	n	e
9	M	1972	12/87	4/90	3	3	85c	85c	13	14	g	h
10	F	1978	9/83	4/90	6	5	85c	85c	15	16	l	m
11	F	1970	9/83	9/90	3	3	46c	46c	17	17	b	b
12	M	1963	9/83	7/91	13	13	45c	45c	18	19	p	p2
13	M	1974	3/82	6/90	3/13	3/13	45c	45c	20	20	q	q
14	M	1974	11/83	4/90	16	16	45c	45c	21	21	r	r1
15	F	1965	2/84	7/91	15	15	74c	74c	22	22	e	e1
16	M	1969	12/85	7/91	1/3	1/3	78d	78d	23	24	n	n1

No	Gender (M/F)	Year of birth	Date isolation Strain 1	Date isolation Strain 2	Serotype Strain 1	Serotype Strain 2	Pyocin Strain 1	Pyocin Strain 2	AP-PCR Strain 1	AP-PCR Strain 2	PFGE Strain 1	PFGE Strain 2
17	F	1985	2/86	8/90	6	6	84c	84c	25	25	u	u
18	F	1973	8/85	1/91	6	6	54c	54c	26	27	v	v1
19	F	1966	10/84	4/91	6	6	85c	85c	28	29	w	e
20	F	1971	4/84	5/90	13	13	82d	85d	30	31	y	z
21	F	1964	12/81	7/91	11	11	11d	NT	32	33	o	t
22	F	1967	9/85	7/91	1/3	1/3	NT	NT	34	35	x	x1
23	M	1978	6/85	7/90	3	3	NT	NT	36	36	D	D
24	M	1985	1/86	1/91	1	1	NT	NT	37	37	E	E
25	M	1978	6/85	1/91	1/3	1/10	NT	NT	38	38	F	F
26	F	1984	3/86	7/91	1	1/6	NT	NT	39	39	G	G1
27	F	1968	10/85	4/91	NT	NT	85c	85c	40	40	NT	NT
28	F	1964	4/85	1/91	3	NT	74c	74c	41	41	e	e
29	F	1969	1/84	7/91	6	3	66c	NT	42	43	K	L

Note: All typing results are given in separate columns, each column displaying data for both strains (numbered 1 and 2) from all individual patients. Isolation dates are given in month/year. Sero-, pyocin-, PCR- and PFGE-types are explained in the Material and Methods section. NT: non typeable.

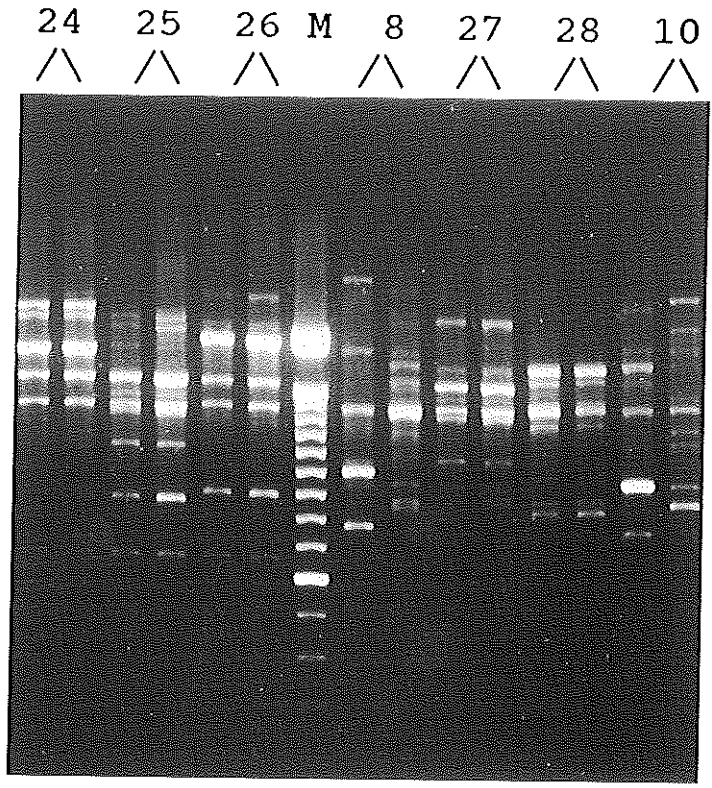

Figure 1: DNA of fingerprints obtained after AP-PCR with primer 1290. Strain designation are indicated above the lanes and are identical to those described in table 1. Genotypically similar isolates were obtained from patient 24, 25, 26, 27, 28. The strains from patient 8 and 10 show different AP-PCR fingerprints, which in these cases is in agreement with PFGE data (see table 1). For the strains from patient 27 useful AP-PCR fingerprints are generated, despite the fact that PFGE proved unsuccessful.
M, molecular mass marker.

DISCUSSION

In previous evaluations of typing schemes for *Pseudomonas* spp. it has been demonstrated that the single genetic approach that was validated, on these occasions the analysis of restriction fragment length polymorphism (RFLP), displayed a relatively high degree of resolution and reproducibility (3, 8, 13). In the present study we demonstrate the lower resolution of phenotypic procedures when compared to DNA typing. This is in agreement with the results of a previous study, where it was demonstrated that analysis of a hybridization-mediated *tox*A RFLP detection was superior to phenotyping. The phenotypic procedures displayed lower typeability and discriminatory power. Another problem with phenotyping methods is the spontaneous conversion of *P. aeruginosa*, which can invalidate reproducibility (13). PFGE and AP-PCR generally show concordant data, thereby strengthening the clinical conclusions that can be drawn from the experimental results. It has to be emphasized, however, that AP-PCR resolves the two isolates of four patients (12, 16, 18 and 22; see Table 1), whereas PFGE considers these strains to be identical. This is not due to the technical inadequacy of AP-PCR, since no run-to-run variability was observed and typing of numerous colonies of a single strain always generated sets of identical fingerprints. A major reason for the discrepancy may be a paper one: the lack of standardization of the rules applied for the description of (sub)clonal relationships. Additional, multicentered validation studies, as were performed for *S. aureus* for instance (14), are urgently needed in this respect. The conclusion from Mouton et al (15) that long-term administration of antipseudomonal antibiotics to chronically colonized CF patients is associated with the development of resistance was confirmed for the pairs that were identical with AP-PCR and PFGE (results not shown).

In recent years a number of large-scale typing studies on *Burkholderia cepacia*, also from CF patients, have been published (16-18). These studies demonstrate that person to person transmission of *B. cepacia* does occur, but that the frequency with which this happens depends on the clinical setting. When patients are well isolated, e.g. in lung transplantation units, patients remain permanently colonized by a single strain (18). When frequent and unobstructed personal contacts are allowed, increased transmission is observed. This implies that the CF patient, encountering different environmental strains during day to day life, will continuously take up those strains that fit in best in his or her lung ecosystem. Ultimately, colonization will

be limited to the occurrence of a single bacterial clone (13). In our patient group, six individuals harbored PFGE type e *P. aeruginosa* at a certain point in time. The widespread occurrence of this genospecies is reminiscent of the general spread of another genospecies in the German Hannover region (13). Further analysis of potential geographic clustering of strains requires additional investigations. The results of this study showed that nine patients underwent a shift in the colonizing *P. aeruginosa* strain as measured by AP-PCR. Since the primary goal of the present study was to position the different typing procedures, it is currently not clear whether this is an accurate or biased reflection of the actual clinical situation. The strains were arbitrary chosen and more detailed studies are needed. We are currently typing all the longitudinal *P. aeruginosa* isolates from families of CF patients known in our hospital. Data from such studies will shed light on colonization convergence and cross-infection in and between the family members.

Presently, a PFGE defined subclone may differ in the position of up to five bands with the clonal type, whereas in case of AP-PCR a single band difference led to the definition of another type. These widely differing approaches for clone/subclone description may be an explanation for differences encountered between PFGE and AP-PCR data as shown in this present study. Current research is focused on determination of subclonal variants of AP-PCR patterns in comparison with PFGE types and subtypes. The validity of this type of research is underscored by a recent publication (19), where AP-PCR is brought forward as an excellent first-line typing procedure, especially where large numbers of strains need molecular typing.

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Chapter 3

Comparative typing of *Pseudomonas aeruginosa* by random amplification of polymorphic DNA or pulsed-field gel electrophoresis of DNA macrorestriction fragments

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ABSTRACT

Eighty-seven strains of *Pseudomonas aeruginosa* were typed by random amplification of polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE) of macrorestriction fragments. Strains were clustered on the basis of interpretative criteria as presented previously for the PFGE analysis. Clusters of strains were also defined based on the basis of epidemiological data and subsequently reanalyzed by RAPD. It was found that in an RAPD assay employing the enterobacterial repetitive intergenic consensus sequence ERIC2 as a primer, single band differences can be ignored: in this case clonally, related strains could be grouped as effectively and reliably as with PFGE. These data could be corroborated by the use of other primer species. However, some primers either showed reduced resolution or, in contrast, identified DNA polymorphisms beyond epidemiologically and PFGE-defined limits. Apparently, different primers define different windows of genetic variation. It is suggested that criteria for interpretation of the ERIC2 PCR fingerprints can be simple and straightforward: when single band differences are ignored, RAPD-determined grouping of *P. aeruginosa* is congruent with that obtained by PFGE. Consequently, this implies that RAPD can be used with trust as a first screen in epidemiological characterization of *P. aeruginosa*. The ability to measure the rate of molecular evolution of the *P. aeruginosa* genome clearly depends on the choice of restriction enzyme or primer when RAPD or PFGE, respectively, is applied for the detection of DNA polymorphisms.

INTRODUCTION

Molecular typing of microbial pathogens is of pivotal importance in the elucidation of transmission routes. By closely monitoring genetic variability, phylogenetic distances can be measured, and these data can give insight into the interrelationship of bacterial, protozoan or fungal isolates (17). Detailed genetic analysis at the species level gives insights into the variability within a bacterial population and generates evidence on genome plasticity and evolution, which in turn leads to bacterial adaptation to various environmental conditions. This type of information can be used in clinical settings to discriminate ongoing epidemics of infectious agent from an incidentally increased infection rate. Various molecular strategies have been adapted to an experimental format such that the data obtained can help the clinical microbiologist to indicate potential risk factors and to track down sources of epidemic strains (16). Besides the technical point of view, several major questions still exist, however. Firstly, there is no general agreement on the optimal typing strategy to be used for a given pathogen (31, 32). Secondly, although there is a general concordance among typing procedures when comparative analyzes are performed, sometimes discrepancies are obvious (22). The assessment of such discrepant results seems to be possible only when further molecular details about the respective organisms are made available. It has been suggested that combining data obtained by differing typing procedures will give optimal insight into strain relatedness (32). However, only a small number of studies describe in detail the basis of the variability observed between different typing techniques.

The aim of the present study was to determine to what degree two frequently used genetic typing procedures give concordant results, using clinical and environmental strains of *Pseudomonas aeruginosa*. Since standardization of restriction site variation, as detected by pulsed-field gel electrophoresis (PFGE) and annealing site variation in random amplification of polymorphic DNA (RAPD) has not been discussed before, sets of clonally related and unrelated isolates of this opportunistic bacterial pathogen were compared in detail.

MATERIAL AND METHODS

Bacterial strains: Strains were selected on the basis of their PFGE-determined genotypes, the determination of which has been described in previous publications (24, 25, 27) (see below for technical details and Table 1 for a survey of strain characteristics). Four groups of strains were gathered. Firstly, the entire Z group (n=24) belonged to a single clonal type (PFGE C-type). PFGE banding patterns differed by up to six new restriction fragments. Strains derived from environmental and clinical sources and various subtypes were represented. The G group (n=16) was comprised of clearly differing strains, seven different ATCC strains were included as well. PFGE banding patterns displayed gross differences, always exceeding the minimum number of six differently oriented DNA restriction fragments. One of the strains in this group was identical to a member of the Z group (internal control duplicate). In the R group (n=25) several small clusters of identical pairs or triplets were mixed. The strains in this group were epidemiologically unrelated but showed similar PFGE patterns. Finally, the B group (n=22) contained several sets of strains with identical PFGE patterns.

Table 1 Compilation of PFGE and RAPD typing data for strains of *P. aeruginosa* [a]

Strain no.	PFGE type	Source of isolation [b]	Date of isolation (mo/yr or yr)	RAPD type (ERIC2/1290) [c]
Z1	C	P8, CF, Hannover	8/86	A/A
Z2	C1	P8, CF, Hannover	4/87	A/A
Z4	C3	P8, CF, Hannover	11/89	A/A
Z6	C5	P9, CF, Hannover	11/87	A/A
Z7	C6	P9, CF, Hannover	6/88	A/A
Z8	C7	P10, CF, Hannover	5/92	A/A
Z10	C9	P4, CF, Hannover	4/86	A/A
Z11	C10	P4, CF, Hannover	4/87	A/A
Z12	C11	P11, CF, Hannover	Not known	A/A
Z13	C12	P11, CF, Hannover	7/84	A/A
Z14	C13	P12, CF, Hannover	1/85	A/A

Strain no.	PFGE type	Source of isolation [b]	Date of isolation (mo/yr or yr)	RAPD type (ERIC2/1290) [c]
Z15	C14	P12, CF, Hannover	12/85	A1/B
Z16	C15	P12, CF, Hannover	3/87	A/A
Z17	C16	P12, CF, Hannover	5/87	A1/A
Z18	C17	clinical environment, Hannover	12/89	A2/A
Z19	C18	clinical environment, Hannover	12/89	A2/A
Z20	C19	P1, CF, Hannover	2/89	A/A
Z21	C20	butchery, tapwater, Muelheim	92	A/A
Z22	C21	river, Muelheim	92	A/A
Z23	C22	swimming pool, Muelheim	92	A/A
Z24	C23	ear isolate, Heidelberg	92	A/A
Z25	C21	river, Muelheim	92	A/A
Z26	C	P8, CF, Hannover	1/86	A/A
Z27	CO	ATCC 33351, serotype 4	Not known	B/C
G1	CP	ATCC 14886, soil	Not known	C/D
G2	CQ	ATCC 33348, serotype 1	Not known	D/E
G3	CR	patient, Heidelberg	Not known	E/F
G4	CS	outer ear infection, DSM 1128	Not known	F/G
G5	C	P8, CF, Hannover (=Z26)	1/86	A/A
G6	AK	burn wound, Hannover	1989	G/-
G7	CT	ATCC 10145, neotype	Not known	H/H
G8	M	P18, CF, Hannover	6/91	I/I
G9	BB	clinical environment, Hannover	12/89	J/J
G10	CU	ATCC 33818, mushroom	Not known	K/K
G11	DM	CF, not from Hannover	1984	L/L
G12	PAK	reference lab strain, Hannover	Not known	M/H
G13	PAO	reference strain, wound, Melbourne	1955	N/-

Strain no.	PFGE type	Source of isolation [b]	Date of isolation (mo/yr or yr)	RAPD type (ERIC2/1290) [c]
G14	CV	ATCC 15691	1950	O/M
G15	CW	ATCC 21776, soil, Japan	Not known	P/N
G16	CX	ATCC 33356, serotype 9	Not known	Q/-
B1	I	P6, CF, Hannover	1/90	R/O
B2	I	P6, CF, Hannover	1/90	R/O
B3	I	P6, CF, Hannover	11/90	R/O
B4	I	P6, CF, Hannover	3/91	R/O
B5	I	P6, CF, Hannover	3/91	R/O
B6	I	P6, CF, Hannover	9/91	R/O
B7	I	P6, CF, Hannover	9/91	R/O
B8	C	P9, CF, Hannover	4/89	A/P
B9	C	P9, CF, Hannover	7/89	A/Q
B10	C	P9, CF, Hannover	8/90	A/-
B11	F	P2, CF, Hannover	8/90	S/-
B12	C	P9, CF, Hannover	8/90	A/P
B13	C	P9, CF, Hannover	5/91	A/Q
B14	G5	P3, CF, Hannover	10/89	T/R
B15	G5	P3, CF, Hannover	7/90	T/R
B16	G5	P3, CF, Hannover	2/91	T/R
B19	F	P2, CF, Hannover	8/90	S/S
B20	C	P9, CF, Hannover	8/90	A/-
B21	F	P2, CF, Hannover	3/91	S/S
B22	CY	brass tube	92	U/T
B23	CY	sink, private household	92	U/T
B24	CY	sink, private household	92	U/T
R10	M3	clinical environment, Hannover	11/93	V/W
R19	M3	clinical environment, Hannover	12/89	W/b

Strain no.	PFGE type	Source of isolation [b]	Date of isolation (mo/yr or yr)	RAPD type (ERIC2/1290) [c]
R24	M5	clinical environment, Hannover	12/89	W/d
R11	K	P11, CF, Hannover	7/85	X/X
R20	K2	P11, CF, Hannover	2/91	X/C
R3	J10	pond, Muelheim	92	Y/P
R12	J1	P7, CF, Hannover	9/85	Y/Y
R21	J8	P7, CF, Hannover	7/89	Y/Y
R25	J7	P12, CF, Hannover	11/89	Y/Y
R5	A	P13, CF, Hannover	12/85	Z/R
R14	A	P13, CF, Hannover	1/86	Z/Z
R7	AH	P24, CF, Hannover	84	a/T
R16	AH	P23, CF, Hannover	84	a/Z
R22	AH	P4, CF, Hannover	84	a/R
R6	B	P15, CF, Hannover	5/86	b/S
R15	B1	P15, CF, Hannover	12/86	b/a
R8	F	P2, CF, Hannover	2/85	S/S
R17	F2	P2, CF, Hannover	5/92	C/d
R23	F	P2, CF, Hannover	5/92	S/S
R13	CZ1	clinical environment, Muelheim	92	d/-
R4	CZ	sink, private household, Muelheim	92	d/Q
R9	G	P3, CF, Hannover	4/86	T/V
R18	G4	P3, CF, Hannover	5/92	T/V

[a] Subtypes are indicated by affixed Arabic numbers, in case of PFGE, this may be reminiscent of differences in the position of six DNA fragments, while in case of RAPD, the cut off was at more than a single band difference. In each of the RAPD tests a single primer was included (either ERIC2 or primer 1290).

[b] For the source of isolation, patients are identified by a capital P; patients 8, 9, and 10 are siblings. All strains, except for the reference and ATCC strains, derive from Germany.

[c] -, not done

RAPD analysis: DNA was isolated according to the Celite affinity chromatography protocol as described previously (4). The DNA was stored in a buffered solution (10 mM Tris.HCl pH 8.0, 1 mM EDTA) at -20°C. RAPD was performed on 50 ng of template DNA as presented before (23, 33). For each strain of *P. aeruginosa*, two RAPD assays were performed. Either primer ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') or primer 1290 (5'-TA-CATTCGAGGACCCCTAAGTG-3') was employed. Because of the complexity of the R group, several strains from within this cluster were also analyzed with a set of other primers. These primers were ERIC1 (5'-ATGTAAGCTCCTGGGGATTCAC-3'), RAPD1 (5'-GGTTGGGTGAGAATTGCACG-3'), RAPD7 (5'-GTGGATGCGA-3'), 325 (5'-TCATGATGCA-3'), 327 (5'-CCTGCTTTGAACACTCTAATTT-3'), 44 (5'-CGCTACCAAGCAATCAAGTTGCCC-3') and 70 (5'-CATCGTCGC-TATCGTCTTCACCAC-3'). Using this same set of primers, some of the strains defined as PFGE-identical or -related strains were reexamined as well. After electrophoresis in 1% agarose gels, the ethidium bromide stained DNA fragments were photographed with Polaroid equipment. Banding patterns were analyzed by two independent researchers and, (sub)types were assigned on the basis of single or multiple band differences.

PFGE analysis: PFGE was performed as described previously (9, 26). *P. aeruginosa* cells were embedded in agarose blocks and treated with proteinase K, *N*-lauroylsarcosine, and EDTA. Before electrophoresis, the DNA was digested with the restriction enzyme *Spe*I (New England Biolabs, UK) and after PFGE banding patterns were visualized by ethidium bromide staining and then photographed. Interpretation was also performed in accordance with previously determined standards implying that separate types should differ by more than six DNA fragments. Each type was coded with a capital letter, and subtypes were identified by numbers.

RESULTS

PFGE data interpretation: The PFGE patterns for all of the strains were determined in previous studies (24, 27) and are summarized in Tables 1 and 2. Strains from the Z-group were considered clonally related (C type, subtypes as indicated in Table 1), although the individual electropherograms may differ in up to sometimes even six DNA macrorestriction fragments (25). The G-, R- and B-group are more heterogeneous, although clusters of related and sometimes even identical (by PFGE) strains may be discerned (see for instance strains B1 to B7 or B22 to B24).

Table 2 Comparative analysis of PFGE typed (sub)clonally related strains of *P. aeruginosa* by multiple RAPD assays

		RAPD type			
Strain number	PFGE type	ERIC2	RAPD1	325	44
R10	M3	V	A	A	A
R19	M3	W	B	B	B
R24	M5	W	B	B	B
R11	K	X	C	C	C
R20	K2	X	C	C	C
R3	J10	Y	D	D	D
R12	J1	Y	D	D1	D
R21	J8	Y	D	D1	D
R25	J7	Y	D	D1	D
R5	A	Z	E	E	E
R14	A	Z	E	E	E

		RAPD type			
Strain number	PFGE type	ERIC2	RAPD1	325	44
R7	AH	a	F	F	F
R16	AH	a	F	F	F
R22	AH	a	F	F	F
R6	B	b	G	G	C
R15	B1	b	G	G	C
R8	F	S	H	H	G
R17	F2	C	I	I	H
R23	F	S	H	H	G
B21	F	S	H	H	G
R13	CZ1	d	J	J	I
R4	CZ	d	J	J	I
B14	G5	T	K	K	K
R9	G	T	K1	K1	L
R18	G4	T	K1	K1	L

Note: Figures in the PFGE types indicate subclonal relatedness: the number of different bands varies between 1 up to maximally 6. The codes presented for primers ERIC2 and 1290 are in concordance with those given in Table 1. For all of the other primers arbitrary codes are given in letters starting with A.

Integrated analysis of the PFGE and RAPD data: All RAPD derived banding patterns were indexed with capital letters. This is exemplified in Figures 1 and 2, and data are summarized in Tables 1 and 2 in a schematic format as well.

The number and sizes of DNA fragments generated by RAPD are clearly primer dependent. As can be deduced from Figure 1, when the ERIC2 primer is employed, between 8 and 15 DNA fragments ranging from 100 up 2,500 base pairs are synthesized by the *Taq* polymerase. When RAPD1 is used, approximately 17 fragments are generated, while for primer 325,

between 16 and 19 DNA molecules can be seen after electrophoretic separation (data not shown). Sometimes smearing is observed when multiple DNA fragments which differ slightly in length are visible.

The members of the PFGE homogeneous Z-group were shown to generate individually similar RAPD banding patterns. From the banding patterns it was concluded that for the entire group only five RAPD (sub)types could be observed. When the ERIC2 primer was applied, for instance, the two subtypes A1 and A2 differed by the presence or absence of only a single DNA fragment when compared to the A type. This may imply that single band differences in the banding patterns generated in this way do not represent epidemiologically relevant genetic differences among related clusters of strains (see also discussion). The only aberrant strain in the Z-group is Z27, which was included as a control sample in this group (PFGE type CO). The distribution of fragment sizes shown by this strain was similar to that of genuine clone C isolates (24).

Data obtained for members of the G-group corroborated the PFGE findings. Major differences in banding patterns were observed; only in the case of strains G1 and G3 were somewhat similar patterns documented (still differing at two positions, but no subtypes were thus identified) . Both the ERIC2 and the 1290 primers generated concordant results in this respect. Note that the RAPD type for strain G5, which is of the PFGE C-type, is identical to the RAPD types as established for the majority of the Z strains.

As can seen in Table 1, data obtained for the B group by ERIC2 RAPD show excellent agreement with the PFGE codes. All the clusters enclosed are adequately recognized by the ERIC2 typing results. In some instances, the banding patterns generated with primer 1290 identified additional heterogeneity among the related strains (for instance, type f for B8 and g for B9). This indicates that this particular primer may give rise to an overestimation of the actual number of distinct types that can be distinguished in a given collection of *P. aeruginosa* strains. These RAPD fragments may be reminiscent of DNA loci displaying a high speed of alteration due to a high frequency of mutation or rearrangements caused by intra- or inter-strain exchange of genetic material.

The most complex set of data was obtained for the R group of strains. In this group, several (sub)clonally related strains are present, as was determined by PFGE. In Table 2, the data obtained by RAPD are summarized; strains are ordered with respect to the initially assigned

PFGE type. As such, it can be deduced that the ERIC2 RAPD tests are in reasonable agreement with the PFGE data: again, the 1290 fingerprints show more variability. For this reason, other primer species were evaluated for typing efficacy. These experiments resulted in a number of interesting observations. It appeared that application of the primers 70, RAPD7 (which is very well suited for typing of staphylococci (32), 327 and ERIC1 did not generate interpretable results. Either the DNA banding patterns were identical for all strains or no DNA was amplified whatsoever. Data obtained with the primers that could be applied successfully are summarized in Table 2 and illustrated in Figure 2. From these data, it can be concluded that RAPD analysis generates results that compare very well with those obtained by PFGE. The RAPD-based grouping is a clear reflection of the PFGE-related clusters. Dependent on how the data are interpreted, it is evident that PFGE subtypes may sometimes be defined as different clonal types by RAPD. Strains R10, R19 and R24, which are PFGE subtypes M3, M3 and M5, respectively, are grouped into two RAPD types. The ERIC2, RAPD1, 325 and 44 data are precisely concordant; only primer 1290 gives rise to an overestimation of the number of types that can be distinguished. The latter primer also shows overdiscrimination with strains R11 and R20. Also, the latter phenomenon can be observed in some of the other groups displayed in Table 2.

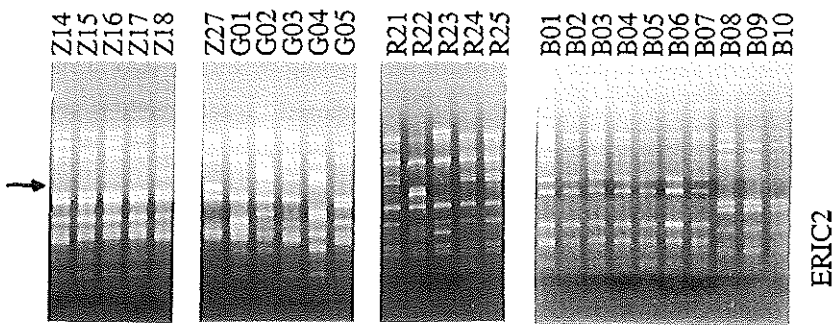

Figure 1: Examples of RAPD generated DNA fingerprints for strains of *P. aeruginosa* genetically clustered in four different groups (Z, G, R and B). The primer used was ERIC2. Strains Z14 to Z18 are part of the clonally related C-cluster as defined by PFGE. Note that only single band differences are observed. The second from the left shows strains belonging to different clonal entities (Z27 to G05): all the banding patterns are clearly different. In the third (R21to R25) and fourth (B01 to B10) panels, some of the epidemiologically clustered strains are on display. Note that B08 to B10 are identical to the C-type strains showed in the panel on the left. For a detailed description of the data see table 1. The arrow on the right indicates a molecular length of 800 basepairs.

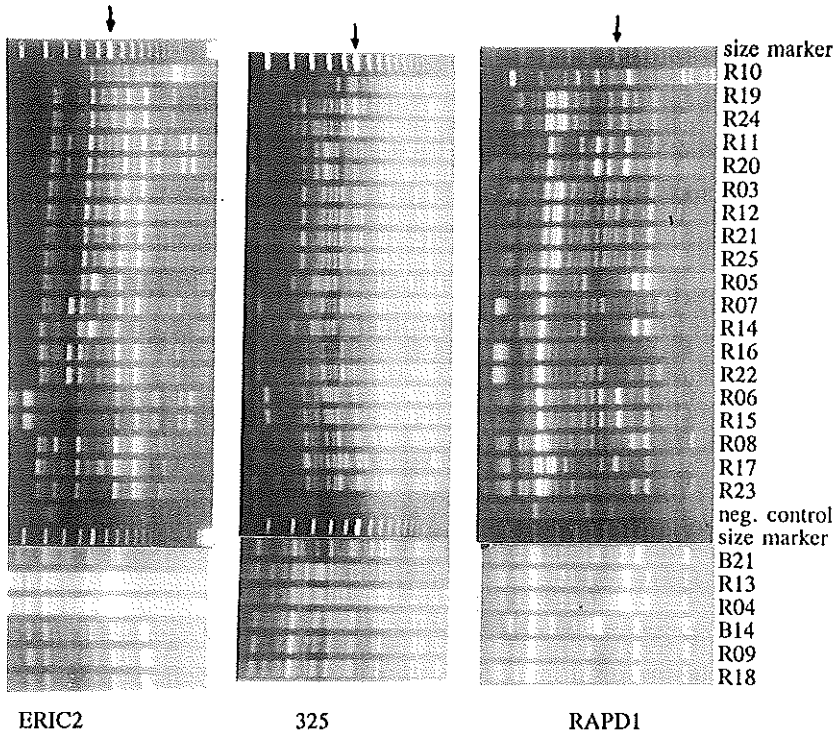

Figure 2: Comparative analysis of the R group strains of *P. aeruginosa* by using different RAPD primers. Clonal relatedness was defined on the basis of PFGE, which is summarized in table 2. Note that the schematic interpretation of data as presented in this figure is given in the same table. Lanes labeled size marker contain 1 kb ladder DNA. The arrow on the left indicates an 800 basepair DNA molecule. In the lane labeled "negative control", amplimers derived from mixtures containing no extraneous DNA were analyzed. Patterns displayed in the panels, going from top to bottom were generated with the help of primers RAPD1, 325 and ERIC2. Note that there is identity among the ERIC2 fingerprints that are shown both in figure 1 and this figure.

DISCUSSION

Pseudomonas aeruginosa is a common pathogen in cystic fibrosis (CF) patients (2, 6, 13). By applying molecular typing procedures, it has been demonstrated that the clinical problems caused by *P. aeruginosa* may result from its capacity to also colonize inanimate surfaces for prolonged periods of time (5, 10). Although the relevance of molecular typing for CF patients may not be as obvious as it possibly should be, several reports of studies employing molecular typing of *P. aeruginosa* were recently published. Striking examples were the proof of existing cross-contamination among neonates in certain clinical settings (34) and a study showing the usefulness of molecular typing in gaining insight in the putative "pseudomonad exchange" between CF patients spending time in summer holiday camps (14). Reservoirs could thus be identified, and the nosocomial ecology of the microorganism could in some instances be unraveled in great detail. In order to perform such studies, the clinical laboratory should have appropriate technical means at its disposal. Presently the number of typing systems described for *P. aeruginosa* is large (19-21, 28, 29), but recently, newly developed procedures such as PFGE of DNA macrorestriction fragments (9, 26, 29) and RAPD analysis (3, 7, 18) have been used for detailed comparisons among clinical and environmental strains of *P. aeruginosa*. However, in only a limited set of studies were the efficacies of the typing strategies compared.

The most elaborate multicentered comparative typing effort for *P. aeruginosa* was presented three years ago (15). This study, which essentially lacked molecular analyzes, suggested that serological typing of the lipopolysaccharides in the outer surface of *P. aeruginosa* provides an efficient means of bacterial typing, especially because it is simple and efficient. CF isolates of *P. aeruginosa* are not-typeable by this method because of their rough phenotype. Due to aberrant phenotypic characteristics, CF strains can be reliably typed only by molecular methods. This was recently confirmed in a study of the colonization of patients with bronchiectasis in which the conventional methods proved ineffective (12). An even more recent study included PFGE typing (11). The experimental results demonstrated that the resolution of PFGE exceeded that of restriction fragment length polymorphism analysis with ribosomal or toxin A DNA probes. Nevertheless, strains of the same type were found in hospitals at different geographic locations. Finally, these authors emphasize that typing data should be interpreted only in the context of sound epidemiological data because, otherwise,

unequivocal conclusions with respect to strain persistence or transmission cannot be drawn. There is a recent (and still singular) publication that discusses the relationship between data obtained by PFGE and that obtained by RAPD for the same set of strains of *P. aeruginosa* (18). The present data indicate that RAPD should serve as a first screen for *P. aeruginosa* typing because of its simplicity and high speed of this technique and that the bacterial grouping results attained coincides with those of PFGE analysis. The authors of reference 18 do not fully discuss the relationship between the two sets of experimental data; neither do they define strict interpretative criteria for the PFGE and RAPD DNA banding patterns. The present communication indicates that if single band differences between RAPD derived fingerprints are ignored, there is excellent agreement of the RAPD results with the PFGE-based grouping of clonally related *P. aeruginosa* strains.

The interpretation of data generated by PFGE was the general subject of a recent and timely discussion (31). In this paper, which tried to define guidelines for the interpretation of the DNA banding patterns in the absence of a generally accepted technologically standardized approach, it was suggested that a difference in the electropherogram of more than three bands should lead to the definition of another, new bacterial clone. Subclones are identified on the basis of smaller numbers of differences. Such a rigid definition does not take into account biological properties such as different degrees of variability in different species. This type of information can be gathered, for example, by studying the results of comparative physical mapping of bacterial genomes (8) and should be included in epidemiological evaluations when available. In the present paper, we show that these criteria may vary by microorganism and that measurement of the speed of genomic evolution heavily depends on primer choice or choice of restriction enzyme, respectively, when either RAPD or PFGE is involved. In case of *P. aeruginosa*, the existence of as many as six differences between the PFGE generated DNA banding patterns may not rule out clonal relatedness. This is confirmed by the data obtained with RAPD primer ERIC2, even if very stringent interpretation criteria are used (only single band differences are ignored). Detailed studies of DNA typing and the standardization thereof should involve, in case of the interpretative analysis of RAPD and PFGE, multiple restriction enzymes for PFGE and multiple primers for RAPD. These should be optimized for all of the medically important microorganisms. This would allow the following typing scheme: by screening with RAPD, clonal relatedness can be determined at high speed and relatively low

costs. This would enable clinical microbiologists to unravel most of the nosocomial epidemics. In a second stage, PFGE could be used for confirmation of the RAPD data and for fine-tuning the sanitary or clinical measures already taken on the basis of RAPD data. The primary criterion for the selection of the restriction enzyme to be used for PFGE pretreatment should be the presence of a sufficient number of restriction sites to allow adequate discrimination and resolution.

In conclusion, it can be stated that RAPD provides an excellent first screen for typing of *P. aeruginosa* and this is supported by data obtained by others (11, 15, 18, 31). The interpretation of data obtained with a single primer, as described in this communication, is straightforward: when single band differences are neglected, full concordance with data obtained by PFGE may be expected. This makes interpretation of the experimental results simple, especially when automated analysis is feasible. The application of RAPD in multicentered studies, however, should be subjected to thorough research, since it has been demonstrated before that RAPD, although highly reproducible within a single laboratory, may generate different experimental outcomes when performed in different laboratories (33). Although it was recently demonstrated that ribotyping may be as discriminative as PFGE (1), in the case of large, (inter)national studies, PFGE may still be the method of choice. On the other hand, a simple single primer RAPD test, as described in the present paper, may be amenable to multicentered standardization, especially in the context of epidemiological investigations by reference labs, and requires a lower level of expenditure than PFGE.

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Chapter 4

Exchange of *Pseudomonas aeruginosa* strains among cystic fibrosis siblings

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Res. Microbiol. 1997;148:447-454

ABSTRACT

The molecular epidemiology of *Pseudomonas aeruginosa* infection in cystic fibrosis (CF) siblings was analyzed by DNA fingerprinting using arbitrary primed polymerase chain reaction (AP-PCR). A total of 306 strains collected from six pairs of siblings over a period of 20 - 126 months (median 64) was studied. Fifty-four different *P. aeruginosa* genotypes were recognized. Two out of six pairs of siblings were ultimately colonized by identical strains, and it was shown that a single *P. aeruginosa* clone can persist in an individual patient for over ten years. No overlap in *P. aeruginosa* genotypes was encountered between families, whereas in all families at least transient cross-colonization with the same genotype was observed. This finding demonstrates that *P. aeruginosa* cross-infection or acquisition of the same strain from an identical environmental source exists within the family situation, but does not always result in a long-term colonization by identical genotypes in all family members suffering from CF.

INTRODUCTION

Pseudomonas aeruginosa is an organism commonly occurring in soil, water, plants, animals and humans. Normally *P. aeruginosa* is a resident of the intestinal tract in a rather small percentage of healthy individuals. It is found sporadically in moist areas of the human skin and in the saliva (12). It can multiply in almost any moist environment and has minimal nutritional requirements. Moreover, it is tolerant to a wide variety of physical conditions. Consequently, the microorganism can be found frequently in the hospital environment and home reservoirs such as sinks, floors, baths, soap-dishes and dishcloths (6). Previous environmental sampling resulted in frequent isolation of *P. aeruginosa* and *P. putida / fluorescens* from various sites in the houses of cystic fibrosis (CF) and non-CF patients (14). Another study documented carrier states exclusively in patients with CF and not in healthy members of the same family (11). In a regional study, Römling et al. (19) identified two separate clones of *P. aeruginosa* in CF patients as well as in moist habitats within the hospital environment. Environmental *P. aeruginosa* isolates from moist and aquatic habitats in Germany apparently contained variants of the same CF clones. This is in contrast with the results of a study in a Danish cystic fibrosis centre, where none of the patients harboured strains similar to those present in the environment (24). From a clinical perspective, colonization with *P. aeruginosa* in CF patients is a common problem, but the original source of the organism and the precise mode of transmission generally remain unresolved (8,9). Although cross-infection of *P. aeruginosa* in cystic fibrosis siblings is often described (1,3,5,9,18,22, 23), the existence of cross-infection in unrelated CF patients remains a point of discussion (8). Several studies showed identity between isolates from unrelated patients (3,7,13,15,19,20,22), but others did not (1). The objective of the present study was to characterize the genetic polymorphism of multiple *P. aeruginosa* strains isolated from six pairs of CF siblings followed up for periods of up to 10 years.

MATERIALS AND METHODS

P. aeruginosa isolates were collected, from 12 different CF patients belonging to 6 families, during hospitalization and/or follow up visits. Isolates were included in the study when a. 2 CF patients originated from a single household, b. both patients were colonized for a minimum period of 20 months and c. at least 2 strains were still available for genetic typing. All patients were frequently analyzed for microbial colonization of the lungs. Thus, 306 *P. aeruginosa* sputum isolates collected from six pairs of CF siblings by the Department of Medical Microbiology & Infectious Diseases of the University Hospital Rotterdam (the Netherlands) were studied. Strains were isolated by standard microbiological procedures and stored in a viable state in glycerol containing media at -70 °C. If two or more morphologically different *P. aeruginosa* strains were cultured from a single sputum specimen, all morphotypes were included in the study. The 306 strains were characterized by arbitrary primed polymerase chain reaction (AP-PCR) with primer 1290 (5'-TACATTCGAGGACCCCTAAGTG-3') as described before (16). Banding patterns were interpreted by visual inspection by two independent observers and all fingerprints that differed by more than one single band were assigned a letter. Differences in ethidium bromide staining intensities were ignored.

RESULTS

Cultivation: The patients' ages and the period of isolation of *P. aeruginosa* can be deduced from Table 1 for all of the CF patients included in the present study. The *P. aeruginosa* isolates were obtained over a period of 20-126 months (median 64).

Table 1 Results of cultivation of all sputum specimens from 6 pairs of siblings.

no.	G	Year of birth	*P. aeruginosa* no. of isolation	sampling period	*Pseudomonas* for AP-PCR	geno-type	no. of isolates	period of persistence (months)
1	♀	1970	188	1184-0193	66	A	3	24
						B	4	28
						C	9	27
						D	1	
						E	47	44
						H	1	
						G	1	
2	♂	1973	50	0791-0793	9	E	7	24
						C	1	
						A	1	
3	♀	1982	25	0287-1292	2	A	1	
						B	1	
4	♂	1980	13	0288-0693	4	C	2	7
						B	2	4
5	♀	1978	97	0983-0394	33	A	4	98
						O	24	126
						P	1	
						Q	2	22
						R	1	
						S	1	

no.	G	Year of birth	*P. aeruginosa* no. of isolation	sampling period	*Pseudomonas* for AP-PCR	geno-type	no. of isolates	period of persistence (months)
6	♀	1974	226	0883-0893	78	A	15	80
						B	1	
						C	1	
						D	2	2
						E	1	
						G	51	69
						F	1	
						H	1	
						I	1	
						J	1	
						K	1	
						L	1	
						O	1	
7	♂	1976	10	0987-0590	2	D	2	32
8	♀	1972	94	1181-0990	55	A	47	62
						B	1	
						C	1	
						D	2	16
						E	1	
						F	1	
						G	1	
						H	1	
9	♂	1986	4	1189-0791	4	A	2	10
						B	2	6
10	♂	1983	38	0589-0793	13	A	9	44
						B	3	29
						C	1	
11	♀	1975	14	1191-0893	10	A	1	
						B	4	20
						C	4	18
						D	1	
12	♀	1972	77	0890-0793	30	D	15	35
						C	13	25
						B	2	6

The other bacterial species isolated from the CF patients were mainly *Staphylococcus aureus* and, incidentally, *Haemophilus influenzae, Acinetobacter anitratus, Escherichia coli, Proteus mirabilis, Streptococcus pneumoniae, Stenotrophomonas maltophilia* and *Enterobacter* spp. In several instances, strains from the fungal and yeast species *Aspergillus fumigatus* and *Candida albicans* were isolated.

Colonization dynamics in the pairs of CF siblings: In the present study, 34 % of the sputum samples (n = 216) yielded 2 or 3 morphologically different *P. aeruginosa* types. However, in 63 % of these cases the morphotypes were genotypical indistinguishable. This can be explained by the fact that morphotype variability as for instance caused by lipopolysaccharide changes, can be caused by minor genetic events that go undetected by gross genotyping procedures such as AP-PCR. It has been demonstrated before that CF populations of *P. aeruginosa* are genetically homogeneous: upon molecular typing of multiple colonies of a single sputum culture genetic identity was revealed (21).

As time progressed, however, most patients harboured several genomic variants. Figure 1 gives a survey of AP-PCR results obtained for the strains derived from one of them; this illustrates the differences in RAPD banding patterns, although persistent colonization by a single genotype is obvious as well (see lane 1-7, 9 and 13-16).

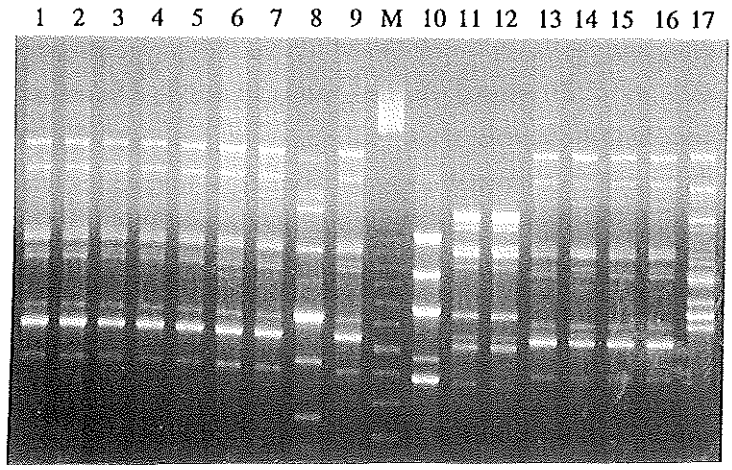
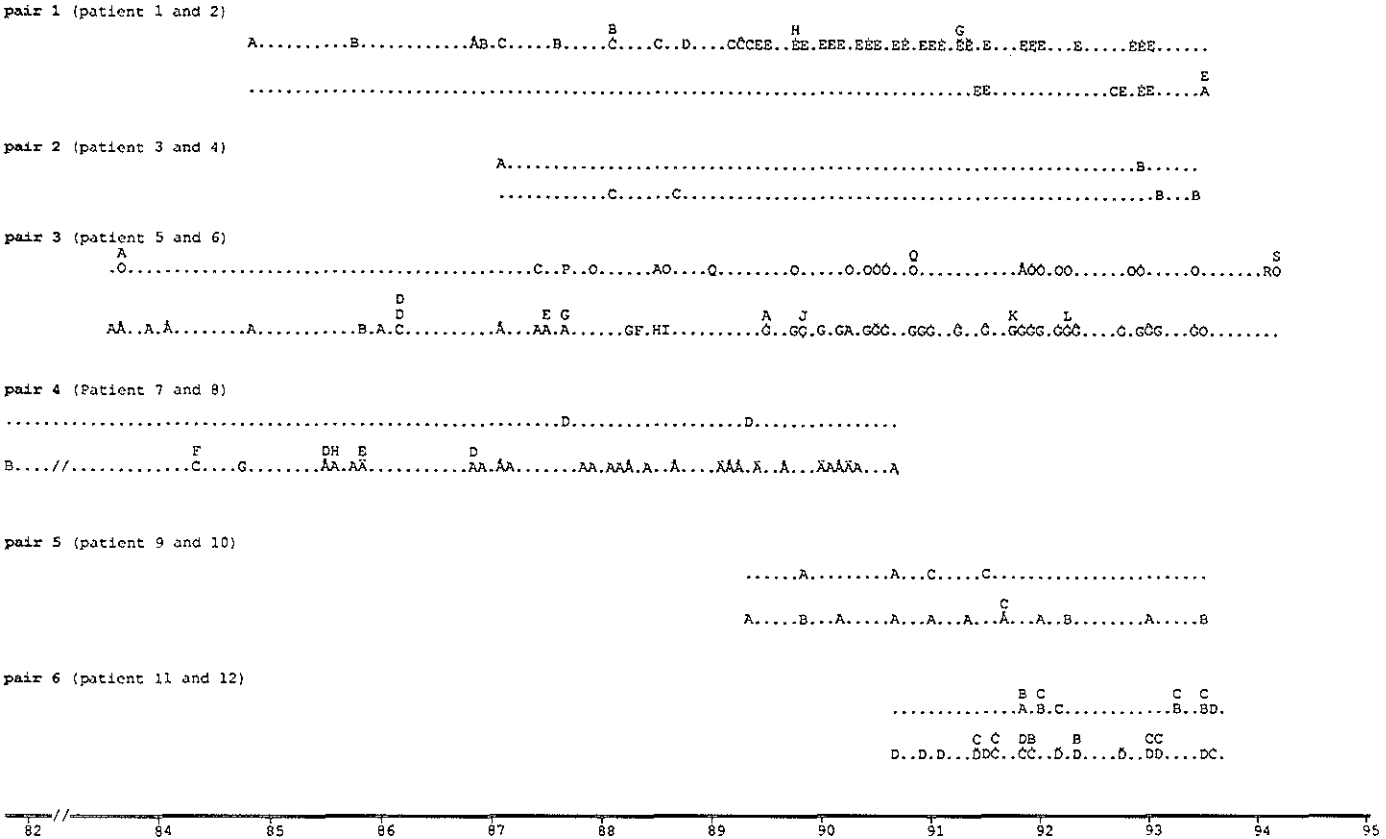


Figure 1: DNA from *P. aeruginosa* was amplified with an arbitrary primer 1290.
Strain designation is indicated above the lanes. The strains are identical to the first 17 strains from patient 6 which are chronologically presented in figure 2. (M : molecular mass marker).

The number of different genotypes varied per patient (Figure 2). In patient 6 for instance, 13 different genotypes were identified (A-L and O, see table 1). Altogether, fifty-four different genotypes could be recognized by AP-PCR fingerprinting. Figure 2 gives a longitudinal survey of *P. aeruginosa* genotypes in 6 pairs of CF siblings. In pair 1, patient 1 showed more variation in the beginning of colonization and became long-term colonized with genotype E. Patient 2 shared the same genotype, and two additional genotypes (A and C) were also shared by this pair. The precise colonization pattern for patient 3 remains unknown because only two strains were available. Patient 4 harboured two genotypes, and the genotype of the last two isolates was identical in both patient 3 and 4. Patients 5 and 6 shared 2 genotypes, but became long-term colonized individually with a different strain; O and G, respectively. These two strains were encountered as potential cross-contaminants in either individual of this pair. Patient 6 initially acquired a strain (genotype A) that was replaced by another strain (genotype G) persisting 71 months. Again, genotype A was found four times, and genotype G was never isolated in the other family member. In patient 7, two identical isolates (type D) were cultured over a period of 20 months. Unfortunately, there were no additional strains available. Genotype D was isolated twice from the other sibling who showed more colonization variation in the early stages and became long-term colonized with genotype A. For patient 9, four isolates were available; two genotypes were identified. Both genotypes were identical to the long-term colonizing strains from patient 10. Patient 10 was colonized with two genotypes of which genotype A was frequently isolated. Patient 11 and 12 shared genotype B, C and D and were colonized with B, C and C, D, respectively. Only patient 8 and, to a lesser extent patient 1 showed more variation in the number of bacterial genotypes *P. aeruginosa* in the early state of colonization. Incidentally, common *P. aeruginosa* genotypes were encountered in all of the sibling pairs. This mainly concerned short-term (cross-over) carrier-ship. Apparently, patients exchange bacterial strains or acquire identical types from common environmental source, but in general, this does not lead to persistent colonization. Only type E in pair 1 and type C in pair 6 are strains that give rise to long term colonization in both individuals in a single household pair.

Figure 2 : Longitudinal survey of *Pseudomonas aeruginosa* genotypes in 6 pairs of CF siblings.



Note: each point represents one month; within each pair each genotype is assigned it's unique letter; if more genotypes of *Pseudomonas aeruginosa* were isolated from one or more sputum samples in one month, it is assigned as different letters, if identical genotypes : Å two isolates; Ā three isolates; Ą four isolates; Ć two isolates; Ċ three isolates; Ď two isolates; Ė two isolates; Ē three isolates; Ę four isolates; Ġ two isolates; Ġ three isolates; Ģ four isolates; Ġ five isolates; Ó two isolates. Note that indices may overlap between pairs but these do not indicate identity (e.g. type A in patient 1 and 2 does not equal type A in patient 5 and 6).

DISCUSSION

CF patients generally acquire *P. aeruginosa* strains in their lungs, and during the course of the colonization the mixture of the strains may vary. Many clinical studies focused on this issue for individual patients (1,5,8,20,22). We present a similar study of the dynamics of *P. aeruginosa* colonization in the CF lung from a family perspective: our analysis aimed at the elucidation of long-term spread of *P. aeruginosa* strains within the family situation. For molecular typing of the strains we used RAPD analysis for several reasons. Firstly, a major problem of phenotyping techniques is the spontaneous conversion of the phenotypic markers, whereas a lower rate of typeability of *P. aeruginosa* occurs. Secondly, in previous studies, AP-PCR and PFGE (pulsed-field gel electrophoresis) displayed superior resolution when compared to sero- and pyocin-typing whereas the performances of PFGE and AP-PCR were comparable (10,16). Thus, it is likely that the presence of genetic variation in strains from the CF patients can be detected accurately and reliably by a single AP-PCR assay (17).

In most patients a predominant genotype became persistent. These results are in agreement with those of other investigators (2,20). Only in patient 2, 3, 8 and 11 the initial genotype was never found again. In six patients who were long-term colonized with one genotype of *P. aeruginosa*, only short-time colonization with other strains was observed. Three patients (10, 11 and 12) were long-term colonized with two genotypes. Interestingly, identity in genotypes was only established when strains from family members were studied. Between families, no overlap in colonizing strains was encountered. These results show that most CF patients ultimately become colonized with a strain which obviously fits best into the micro-environment of their lungs. Although precise records of antibiotic treatment were not available for all of the patients, the genotypic data reveal that antibiotic therapy (which is administered very frequently in these patients) does not induce complete bacterial sterilization of the lungs nor does it enable a new *P. aeruginosa* genotype to take over easily.

Høiby (4) defined colonization to be chronic when cultures of sputum samples yielded *P. aeruginosa* for six consecutive months and/or when two or more precipitins against *P. aeruginosa* were present in the serum. This definition cannot be used for the patients in the current study, since none of them was sampled for six consecutive months after the start of *P. aeruginosa* colonization. Start of chronic colonization according to the criteria postulated by Høiby remains therefore unknown in our patients. However, our study shows that chronicity of

infection should be based upon detailed genetic analyzes of the *P. aeruginosa* isolates. Frequent (dis)appearance of strains seems to be observed (Figure 2). This can be explained by assuming mixed colonization by genotypes that temporarily differ in relative abundancy. During exacerbation periods, a given type may outgrow (but apparently not replace) another type. This of course causes a selection of the predominant type at the cultivation stage. Colonization and infection of CF lungs with *P. aeruginosa* are highly dynamic processes.

In our study, four pairs of siblings acquired a genotypical different long-term colonizing strain. This is in contrast with previous studies (5,9). The results of our study mainly support short-term cross-contamination between most related patients. If environmental sources or cross-infection were the most important origin of infection in the family situation, we would have expected to find a higher colonization rate with identical strains in our pairs of siblings. Probably the clinical state of the respiratory tract and the appropriate strain fitting best in the CF lung's environment are more significant factors for establishing long-term colonization.

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Chapter 5

Molecular epidemiology of *Staphylococcus aureus* strains colonizing the lungs of related and unrelated cystic fibrosis patients

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ABSTRACT

Background: Cystic fibrosis (CF) patients can become persistently colonized with *Staphylococcus aureus*. This is initiated at an early age and may continue until or sometimes even during adolescence. Little is known about the epidemiology and cross-infectivity of *S. aureus* in CF patients, whether via the environmental or person-to-person.

Methods: *S. aureus* strains (n=189), isolated from six unrelated CF patients and six pairs of CF siblings were genetically typed by arbitrary primed polymerase chain reaction (AP-PCR) assays.

Results: This longitudinal study revealed 35 different genotypes among the 189 strains, the median number of types in a patient was three (range 1 - 6). One common *S. aureus* genotype was found in six patients and involved twenty percent of all strains analyzed. Ultimately, in most of the patients long-term colonization with a single genotype was observed. In several, but certainly not all, pairs the siblings became persistently colonized with isolates that could not be discriminated by the typing method used; different *S. aureus* genotypes were isolated on an incidental but relatively frequent basis. Only one pair of siblings never shared identical strains at any moment of time during the screening period.

Conclusions: In five of six cases, identical isolates were shared by CF siblings at a certain time. This suggests intra-familiar transmission or the presence of a common environmental source. The fact that in most of the CF sibling pairs different genotypes of *S. aureus* caused the ultimate long-term colonization indicates that, despite regular cross-colonization, patient characteristics select the *S. aureus* strain best adapted to the affected lung. Some genotypes may be particulary prevalent in the CF patient population, but additional studies are needed to confirm this.

INTRODUCTION

Cystic fibrosis (CF) is caused by mutations in the transmembrane conductance regulator gene and is attended by chronic infections of the airways (1,2). The lower respiratory microflora in young CF patients generally includes *Staphylococcus aureus* and *Haemophilus influenzae* (1), while *Pseudomonas aeruginosa* is the most common pathogen encountered at a higher age. This shift in species prevalence is largely unexplained but it has been documented that *P. aeruginosa* is capable of producing substances that may inhibit the growth of, for instance, *S. aureus* (3). The pathogenicity of *S. aureus* in CF patients has been addressed previously from various experimental perspectives. It has been proposed, for instance, that the bacteria from CF patients may simply attach more firmly to CF mucosal cells (4). Strongly adherent *S. aureus* strains could be selected on the basis of their affinity for CF epithelia, or on the other hand, the local conditions in the CF lung may induce an adherent phenotype. Several *S. aureus* typing studies, however, failed to demonstrate the increased prevalence of a 'CF-associated' types (4,5,6). Capsular types appeared to be randomly distributed and no increased anti-capsule antibody levels were encountered among CF patients (5). Esterase electrophoresis, capsular polysaccharide serology, phage typing and antibiogram determination on several hundreds of French CF-derived *S. aureus* isolates also failed to identify a particular CF- type strain (6).

The long-term colonization of *S. aureus* in cystic fibrosis patients has not been studied in great detail yet. The purpose of the present study was to assess the diversity and variability of *S. aureus* isolates in CF patients and to determine whether transmission in the family situation is a frequent event. The epidemiology of *S. aureus* in CF patients was studied with the application of arbitrary primed polymerase chain reaction AP-PCR (7).

MATERIALS AND METHODS

CF patients and microbiological surveillance: Sputum samples from CF patients were analyzed by standard microbiological techniques. Portions of sputum were washed twice in physiologic salt solutions and inoculated on blood agar or chocolate agar. Longitudinal *S. aureus* isolated from two groups of CF patients were evaluated. The groups were defined as follows: group 1, CF patients who were living in a geographically different area and did not share the same room or ward during their hospitalization; and group 2, pairs of siblings from a single households. The selection criteria for group 1, comprising six unrelated selection criteria for group 1, comprising six unrelated, clinically confirmed CF patients, were: persistent *S. aureus* colonization for more than 6 years, with at least 5 isolates still available for genetic analysis. For group 2, comprising six pairs of CF siblings, the criteria were that both CF patients had been colonized with *S. aureus* for at least 5 months and had more than two isolates available in the archival *S. aureus* collection. At the same time, the culture results of all sputum samples taken from these patients were evaluated.

Bacteria: A total of 189 isolates of *S. aureus* were collected from sputum samples derived from CF patients attending the Department of Pulmonary Disease which were analyzed by the Laboratory for Medical Microbiology (University Hospital Rotterdam, the Netherlands). The *S. aureus* isolates were stored in soy tryptone broth supplemented with 15% (vol/vol) glycerol at -70 °C. To determine the genetic homogeneity of the *S. aureus* populations inhabiting the lungs of a given CF patient, 10 colonies of *S. aureus* isolates encountered in a primary culture of five different sputa were stored as well.

Genotyping: AP-PCR was performed according to the methods as described by Kluytmans et al.(8) and van Belkum et al (9,10). For DNA isolation, bacteria were resuspended in 150 μl of TEG buffer (25 mM Tris, 10 mM EDTA, 50 mM Glucose, pH 8,0) and 75 μl lysostaphin (10 mg/ml; Sigma, St. Louis, Mo.) and incubated for 1 h at 37°C. The subsequent isolation and purification of DNA was performed with 4 M guanidine-isothiocyanate and Celite according to previously described procedures (11). The DNA concentration in the resulting eluates was estimated by gel electrophoresis through 1% agarose gels in Tris-borate-EDTA (TBE) buffer, containing ethidium bromide, and in comparison with samples containing a known amount of bacteriophage lambda DNA. PCR-based DNA fingerprinting analysis was performed with

primer ERIC2: AAGTAAGTGACTGGGGTGAGCG and primer 1: GGTTGGGTG-AGAATTGCACG[10]. The PCR mixtures consisted of 10 mM Tris-HCl (pH 9,0), 50 mM KCl, 2.5 mM MgCl2, 0.1 % gelatin and 1 % Triton X-100, 0.2 mM each deoxyribonucleotide triphosphate, 0.2 U *Taq* DNA polymerase (HT-Biotechnology, United Kingdom) and 50 pmol of the individual arbitrary primer was added. PCR involved 40 cycles of denaturation, annealing and DNA chain extension (1 min at 94°C, 1 min at 25°C and 2 min at 74°C) in a Biomed model 60 thermocycler. Amplified DNA was separated by gel electrophoresis (3 h, 100 mA constant current) in 1 % agarose, containing ethidium bromide, and photographed. The resulting banding patterns were interpreted visually by two independent observers. The combined banding patterns of both AP-PCR tests performs (ERIC2 and primer 1 mediated) were used to identify novel genotypes, which were encoded by letter; the use of capital or lower case letter in the resulting schemes is of no particular significance. Differences in band staining intensities were neglected, as were single band differences.

RESULTS

Microbiology: In total, 616 isolates of *S. aureus* were obtained from six pairs of CF siblings and six unrelated CF patients over a period of 5-148 months (median 82) of which 189 were available for genotyping. The median age at onset of colonization, as determined by the first positive culture was 13.4 years (range 3 - 24). All *S. aureus* strains were methicillin sensitive and, if necessary, CF patients were treated with intra-venous flucloxacillin. This antibiotic treatment was administered two to four times per annum. This type of treatment will affect the microbial flora of the individual CF patient, but data described in this communication (see below) emphasize the fact that this type of therapy does not eradicate entire *S. aureus* populations from the CF lung. Other microorganisms isolated from the CF patients included : *Haemophilus influenzae, Candida albicans, Aspergillus fumigatus, Escherichia coli, Proteus mirabilis, Streptococcus pneumoniae, Stenotrophomonas maltophilia* and *Enterobacter* species. Patients 3, 6, 8, 16 and 17 were mainly infected with *H. influenzae*. Patient 5 was chronically colonized with *E. coli* (Tables 1 and 2).

Genetic homogeneity of the *S. aureus* populations: In general, no differences in morphotypes were observed upon primary cultivation of *S. aureus* from CF sputum samples (see below for a single exception). Similarly, all 10 *S. aureus* colonies selected from cultures of five CF sputa, collected on different occasions from an individual patient, were genotypically identical on the basis of the AP-PCR data. Apparently, CF lungs are inhabited by clonal populations of *S. aureus*.

***S. aureus* colonization patterns in unrelated CF patients:** In group 1, several dynamic *S. aureus* colonization patterns were seen (Figure 1). From patient 1, five different genotypes (t, u, J, K and L) were isolated during the early years of colonization. After 29 months of colonization, a sixth and final genotype (type n) established itself permanently. Patient 4 and 5 were colonized with one dominant genotype each (o and H, respectively), but other genotypes were cultured on occasion (F and G twice and o once). Patients 2 and 6 harbored two (C and y) or three (H, M and I) different *S. aureus* genotypes but the strains initially isolated disappeared and the patients became colonized long-term with a single genotype (H or I) after 25 and 77 months. Patient 3 harbored two different strains, although one genotype was isolated only twice, once at the beginning of the colonization and once at the end of study period after a 97 month interval. This genotype was unique for this patient and this result may reflect persistent colonization, although at low concentrations. Alternatively, this type might represent recurring environmental contaminant or a contaminant from the patients' nasal flora. In the intervening period another genotype, also seen in patient 4 and 5 (genotype o), was isolated six times.

***S. aureus* colonization patterns in pairs of siblings with CF:** In group 2, comparable colonization patterns could be distinguished (Figure 2). Colonization with one major genotype was seen in many of the patients. Incidentally other strains were cultured in a sporadic manner, again mainly during the first years of colonization. Some of the other genotypes were isolated only once, except, for instance, in patient 12, where a genotype was found twice over a period of 3 months (Figure 2). Some of the patients harbored two major genotypes which were isolated frequently (see Figure 2, e.g. patient 8). A clear transition of the early most prevalent genotype (o) to another genotype (i) was noticed in patient 9, and a similar phenomenon was observed in patient 15 and 16. Patient 17 was colonized with one strain initially but this was replaced by four alternately occurring genotypes in the later stages; one genotype (a) was

cultured twice in the same month and two types (l and b) were cultured three times consecutively in a short period of time. Only three of six pairs of siblings mutually harbored identical strains for prolonged periods of time (pairs 1, 3 and 6). In pairs 2 and 5, a strain carried by one of the family members was identified on a single occasion in the other patient; this concerns genotypes i and z. In the other three pairs, different genotypes were usually carried. In conclusion, for five of six sibling pairs, strain sharing was, at least on an occasional basis, observed; however, in some of the pairs the time between isolation of identical AP-PCR type strains was often quite long. This is true not only within individual patients (e.g. patient 18, in whom o-type strains were isolated in 1978, and then again 10 years later in 1988), but also among siblings (type z isolated in patient 16 in 1987, and later, in 1992, in patient 15). This could be due to the lack of sensitivity of microbiological sputum analysis or very small numbers of bacteria present in the patients'lungs. Then again, as already discussed above, patients may share an environmental source of *S. aureus*.

Thirty-five unique *S. aureus* genotypes were recognized among the patients described in this communication. From Figures 1 and 2 it can be observed that some of the AP-PCR typed isolates were present in multiple patients. However, most of the strains occurred in single patients only (23 out of 35 types, 66%), thus giving rise to unique strain - patient combinations. Six types (a,b,i,z,F and G) occur in two related patients; The genotypes a,b,F and G were each encountered in a single, non-related patient as well. Genotypes n, C and E were each found in two CF patients, whereas y and H were identified in three patients. Genotype o was undisputably the most prevalent type; this type was found in the lungs of six out of 18 patients, two of whom formed a family pair.

Table 1 Summary of sputum microbiology for six unrelated CF patients.

PT	G	Year of birth	Total no. of cultures	*S. aureus* number	isolates period	*P.aeruginosa* number	isolates period	others number	species period	negatives number	culture period
1	♀	1973	51	29	03/86-02/94	38	05/88-12/94	44	03/86-06/94	1	09/86
2	♂	1964	68	64	02/84-12/94	1	0887	14	05/85-06/94	1	11/91
3	♂	1966	97	51	10/84-02/92	63	06/89-02/92	55	11/84-04/91	1	09/85
4	♂	1970	59	55	01/84-12/94	5	10/90-01/91	6	12/84-03/90	1	10/89
5	♂	1960	106	57	02/84-05/94	6	09/84-10/93	80	02/84-10/94	20	03/85-07/94
6	♀	1969	61	57	01/84-11/94	40	04/90-11/94	20	01/85-11/94	1	07/93

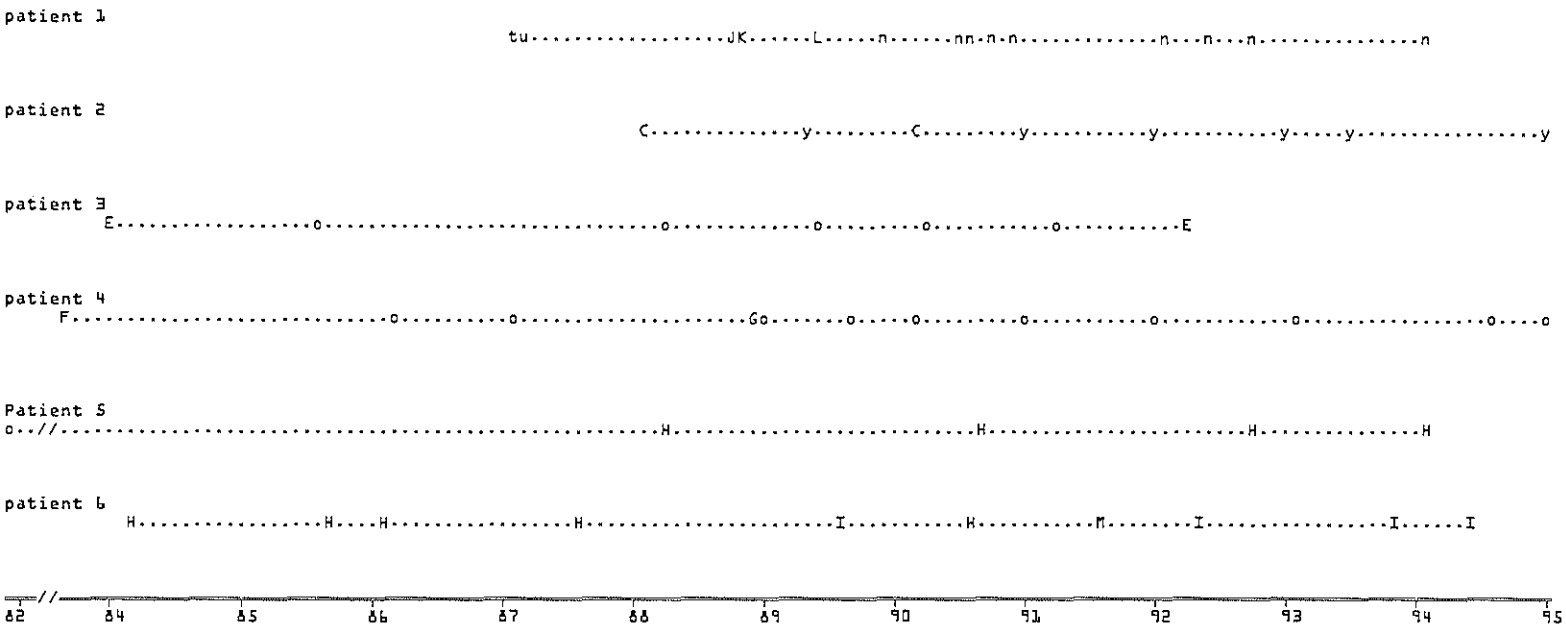
Note: Patients 3 and 6 were colonized for a long period with *H. influenzae*, and patient 5 with *E. coli*. In most patients overlap occurred in periods of *S. aureus* and *P. aeruginosa* colonization.

Table 2. Summary of sputum microbiology for six pairs of siblings with CF.

pair	PT	G	Year of birth	Total no. of cultures	*S.aureus* number	isolates period	*P.aeruginosa* number	isolates period	other numbers	isolates period	negatives number	isolates period
1	7	♀	1970	144	12	06/86-03/90	188	11/84-01/93	12	11/83-02/90	9	05/84-11/91
1	8	♂	1973	60	32	11/84-10/92	50	04/91-11/94	22	11/84-10/92	0	
2	9	♀	1982	63	44	04/85-08/94	25	07/91-08/94	14	10/84-08/94	2	05/86-04/89
2	10	♂	1980	48	12	01/88-09/94	13	09/84-09/94	12	09/84-04/94	19	07/84-06/94
3	11	♀	1974	165	14	05/87-10/89	226	12/83-03/94	13	08/83-04/91	3	12/83-10/89
3	12	♀	1978	73	16	03/86-01/95	97	04/84-12/94	2	10/86-05/87	4	11/90-12/90
4	13	♀	1967	135	24	04/85-11/89	151	10/86-09/94	13	04/85-12/90	21	01/84-02/91
4	14	♂	1962	24	25	09/85-12/94	0		8	01/91-11/94	1	11/90
5	15	♀	1977	12	9	11/84-12/93	1	09/89	4	02/92-12/93	2	10/87-09/90
5	16	♂	1972	41	24	11/84-12/94	6	01/85-02/88	19	03/81-12/94	3	01/85-03/88
6	17	♂	1971	101	71	01/85-12/89	2	01/85-09/88	70	01/85-05/89	9	07/85-11/89
6	18	♀	1973	19	19	01/78-03/91	0		17	01/85-05/90	1	10/88

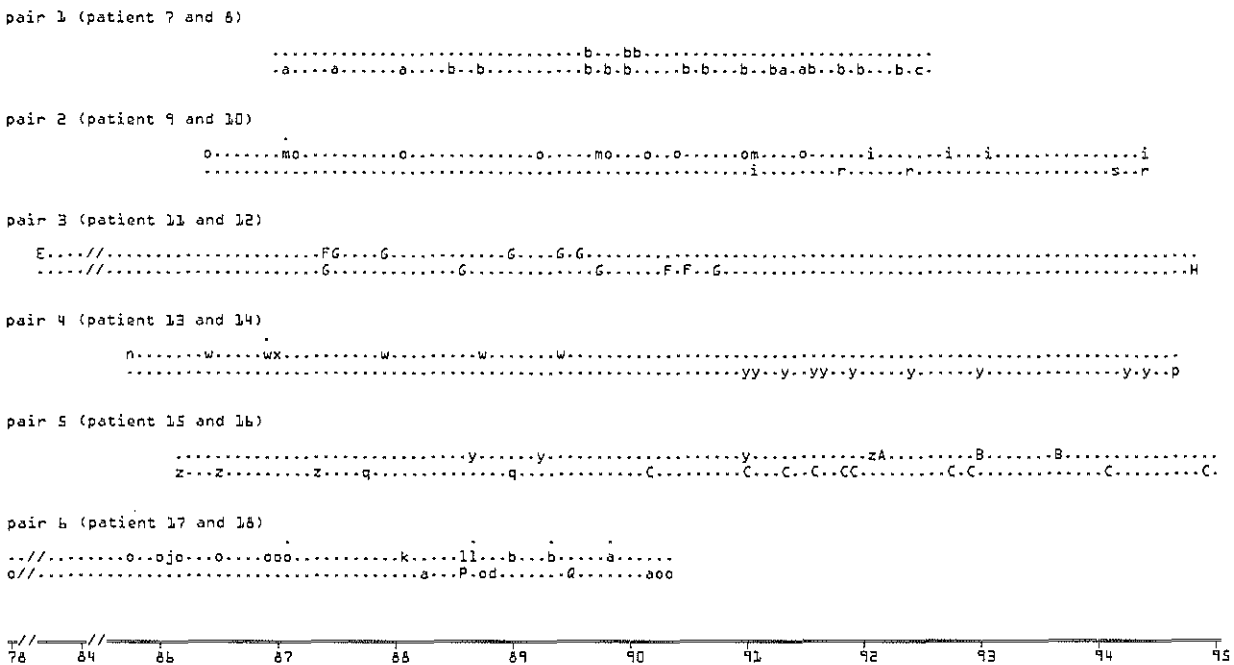
Note: Patients 8, 6 and 17 were colonized for extended periods with *H. influenzae*. In most patients overlap occurred in periods of *S. aureus* or *P. aeruginosa* colonization.

Figure 1 Longitudinal survey of *Staphylococcus aureus* genotypes in 6 unrelated CF patients.



Note : Each genotype is characterized by a capital or small letter. A small dot in the patient related lines corresponds with a period of one month.

Figure 2 : Cultivation of different *Staphylococcus aureus* genotypes in 6 pairs of CF siblings.



Note: Each genotype has is characterized by a small or capital letter; subclones are identified by an affixed number; a small dot above a letter indicates that the strain involved was isolated twice in the same month: a point on the horizontal, patient related lines indicates a period of one month.

DISCUSSION

In general, staphylococci are cleared from the respiratory tract by enclosure within the mucous gel and ciliary action. In CF, the mucus changes in viscosity and this lead to enhanced possibilities for staphylococci to successfully achieve long-term colonization. It has been suggested that other changes in micro environment such as elevated levels of inducing compound, e.g. calcium or mucin (12), may increase staphylococcal adherence. In the present longitudinal study most CF patients had periods of long-term colonization by one predominant *S. aureus* genotype. The use of AP-PCR allows adequate resolution of different *S. aureus* genotypes (8,9,10). Variation in *S. aureus* genotypes within single patients is observed but the pattern is less complex compared to that was observed previously for *P. aeruginosa* (12).

The epidemiology of airway colonization with *S. aureus* in CF has been investigated previously with the aid of pulsed-field gel electrophoresis (14). Only in 6 of 34 CF patients colonized with *S. aureus* exhibited persistence of the same molecular type; in three patients cycles of carriage and loss of strains and appearance of new strains were observed. The colonization patterns found in the present study are in agreement with those observed by Brancher et al (6), who used esterase identification, capsular polysaccharide typing and phage typing for strain tracking. Replacement of a predominant clone was seen in only five of 18 patients. From data described in the present paper and in previous publications, it appears to be likely that in the individual patients' bronchial tree circumstances exist that, in combination with adherence (and possibly other) factors of *S. aureus*, determine which strain will ultimately colonize these patients for prolonged periods of time.

Assuming that selection of 10 *S. aureus* colonies from a primary culture plate gives an adequate cross-sectional representation of population in the sputum, it is interesting to note that all colonies were apparently clonally related, if not genetically identical. The fact that no mixtures of strains were encountered indicates that *S. aureus* isolates are capable of efficiently excluding other, competing isolates of the same species. In only one culture were two morphologically different types found, and both displayed an identical genotype (data not shown). Previous studies on *H. influenzae*, in which 48 individual colonies from three primary culture plates of one sputum sample were analyzed genotypically, also demonstrated that all colonies were identical (15).

In some of the six pairs of siblings, identical and persistently occurring strains were isolated (e.g. genotype G in pair 3). Clearly, cross-colonization takes place in the family situation. This is also illustrated by the incidental occurrence of a strain that was persistently present in another family member.

Identical *S. aureus* genotypes were found in related and unrelated CF patients, which is in contrast with our experience with the long-term epidemiology of *P. aeruginosa* from the same CF patients (13). Genotype o was seen in 6 CF patients (33 %) and accounted for 20 % of all tested strains. Additional research is needed to demonstrate if this genotype is typically associated with cystic fibrosis.

In recent literature several of the molecular features underlying *S. aureus* adherence were addressed in detail. Factors as diverse as protein A (16), fibrin or fibrinogen receptors (17) and receptors for various other eukaryotic cell compounds (18) have been implicated in bacterial colonization. The present collection of CF related *S. aureus* strains, both the more prevalent and the occasionally occurring strains, should enable the detailed analysis of staphylococcal features important in the establishment of a permanently propagating population of *S. aureus* bacteria in the lungs of CF patients.

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Chapter 6

Variable numbers of tandem repeat loci in genetically homogeneous *Haemophilus influenzae* strains alter during persistent colonization in cystic fibrosis patients

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ABSTRACT

Serial sputum isolates of *Haemophilus influenzae* (n = 69) were obtained from eight patients suffering from cystic fibrosis (CF). For two of these patients all strains were analyzed for polymorphism in the major outer membrane protein (MOMP) profile. For all patients the strains were genetically characterized by random amplification of polymorphic DNA (RAPD) analysis. All strains were included in a survey for polymorphism in regions containing moieties of repetitive DNA as well. A single locus containing trinucleotide repeat units, three loci harboring tetranucleotides, one region comprising pentanucleotide units and two hexanucleotide repeat unit-containing loci were analyzed for repeat number variability. Most of the regions were previously shown to be directly adjacent to or even within virulence genes. All regions behaved as genuine variable number of tandem repeat (VNTR) loci in the sense that genetic polymorphism based on the presence of varying numbers of repeat units could be demonstrated among different strains. Interestingly, several of the repeats showed variation in the absence of the variability as assessed by major outer membrane protein or random amplification of polymorphic DNA analysis. These observations indicate that the repeat loci may vary independently from major chromosomal polymorphism. Consequently, *H. influenzae* appears to modify its virulence gene regions of the chromosome during persistent colonization of the lung in cystic fibrosis patients.

INTRODUCTION

Cystic fibrosis (CF) is a devastating genetic disease caused by point mutations in the cystic fibrosis transmembrane regulator (CFTR) gene. The mutations generate a lung phenotype enabling persistent colonization by various bacterial species that, in turn, cause most of the CF-associated morbidity and, in the end, mortality. The major CF-associated bacterial pathogens are *Pseudomonas aeruginosa* and *Staphylococcus aureus*, while an organism such as *Haemophilus influenzae* is also frequently encountered as a colonizer of CF-affected lungs (1-3).. Persistent colonization with *H. influenzae* can be detected (4, 5), which emphasizes its putative involvement in CF pathogenicity. The mechanism by which *H. influenzae* bacteria cause pathogenic effects are still enigmatic, however. On the other hand, numerous virulence factors synthesized by *H. influenzae* have been identified, many of which can display phenotypic phase variation. The relationship between phenotype switching and pathogenicity, however, is still unclear and requires additional investigation.

Phenotype switching in *H. influenzae* has been associated with variability at the level of repetitive DNA. The first gene for which this was demonstrated, *lic*1, appeared to be involved in lipopolysaccharide (LPS) biosynthesis (6). The *lic*1 virulence gene was shown to contain a variable number of consecutive copies of the tetranucleotide unit (5'-CAAT-3') within the coding region. Variation in the overall number of repeat units moves ATG initiation codons in or out of the protein synthesis frame. Similar switches were identified in the genes *lic*2 and *lic*3, found in both *H. influenzae* and other species of *Haemophilus* (7-9). Another clear example of DNA repeats acting as molecular switches is provided by a gene encoding a 120 kDa haem-repressible haemoglobin-binding outer membrane protein in which a CCAA repeat was encountered (10). Besides the modulation of open reading frames, repeat polymorphism also appeared pivotal in promoter strength alternation. By changing the distance between the -10 and -35 consensus boxes differential gene expression could be achieved (11).

The correlation between modulation of virulence factor expression and DNA repeat variability was generalized by scanning of the whole genome sequence for *H. influenzae* (12) for the occurrence of tetranucleotide repeats. It was established that these repeats were invariably associated with putative virulence factor-encoding genes (13). Additional studies revealed that other classes of repetitive DNA are also involved in the regulation of bacterial

pathogenicity and the possible clinical importance of this feature was emphasized (14). Elements like these are called variable number of tandem repeat (VNTR) loci, an abbreviation that will be used throughout the current communication.

The present study was undertaken in order to establish whether or not DNA repeats vary in the in vivo situation during persistent colonization of CF affected lung tissue by individual strains of *H. influenzae*. This could have major implications for therapy and pathogenesis, since the potential to vary virulence factor expression may enable escape from immunity or chemotherapy.

MATERIALS AND METHODS

CF patients and bacterial strains: Two groups of CF patients were included in the present study. Two patients from the Academic Medical Center in Amsterdam, identified by the codes CF30 and CF32, were chosen as the source for an initial control group of strains. During the screening period the CF patients suffered from exacerbations caused by *H. influenzae*. The patients were intermittently treated with antibiotics. *H. influenzae* strains (n=18) derived from these patients were grouped on the basis of the major outer membrane protein (MOMP) characteristics and their genotypes were determined by random amplification of polymorphic DNA (RAPD) analysis as described previously (5, 15). From these patients, several MOMP-identical isolates were included (n=3 strains per patient), whereas also several MOMP-diverse strains were selected (n=7 and n=5, respectively). The RAPD patterns were identical for all strains belonging to either of these patients. These strains, which were isolated between May 1990 and January 1993, were used to survey the relatedness between DNA repeat variability and MOMP characteristics as an indicator of genetic relatedness or variability (see Table 1).

Subsequently, serially isolated *H. influenzae* strains were collected from CF patients (n=6) attending the University Hospital Rotterdam. The periods during which strains were collected were relatively long, varying between one and eight years in duration. Fifty-one strains were collected and analyzed with respect to overall genotype by RAPD analysis and with respect to more localized variation by analysis of DNA repeat polymorphism (see Table 2).

H. influenzae strains were cultured from sputum samples which were obtained during one of the regular, routine check-ups of the CF patients. Aliquots of the strains were stored at -80°C in glycerol containing media. Prior to DNA analysis bacteria were cultured on chocolate agar and checked for species homogeneity. Determination and antibiotic susceptibility testing were performed according to standard microbiological methods (16).

Table 1 Analysis of VNTR loci in MOMP-typed isolates of *H. influenzae* colonizing the lungs of cystic fibrosis patients attending the Academic Medical Center in Amsterdam, the Netherlands.

Patient ident.	Strain number	Hi4-3	Hi4-5	Hi4-10	Hi4-11	Hi3-1	Hi5-2	Hi6-1	Hi6-2
MOMP identical, serial isolates									
CF30	14	15	14	13	26/15	9	2	9	3
CF30	16	15	11	12	24/20	9	2	9	3
CF30	17	15	11	14	26/10	8	2	9	3
MOMP variable, RAPD identical									
CF30	19	15	11	12	27/12	9	2	9	3
CF30	20	15	11	11	25/13	9	2	9	3
CF30	21	16	10	11	25/12	9	2	9	3
CF30	22	15	11	12	31/13	9	2	9	3
CF30	23	14	10	11	31/12	9	-	9	3
CF30	24	16	10	11	31/12	9	-	9	3
CF30	25	15	13	12	31/16	9	-	9	3
MOMP/RAPD identical, serial isolates									
CF32	27	2	6	3	20	9	3	11	3
CF32	28	2	6	3	20	9	6	11	3
CF32	29	2	7	3	20	9	6	5	3
MOMP variable, RAPD identical									
CF32	30	2	7	3	19	9	5	11	3
CF32	31	2	14	3	19	9	7	11	3
CF32	32	2	12	3	22	9	8	11	3
CF32	33	2	7	3	17	9	4	11	3
CF32	35	2	7	3	18	9	6	11	3

NB. The patients strains and genotypes have been described previously (5), the figures in the columns under the heading VNTR analyzes define the number of repeat units present in a given locus.

Table 2 Analysis of VNTR loci in serial isolates of *H. influenzae* colonizing the lungs of cystic fibrosis patients attending the Academic Hospital Rotterdam.

Patient	Strain	Isol. date	RAPD type *	Hi4-3	Hi4-10	Hi4-11	Hi3-1	Hi5-2	Hi6-1	Hi6-2
N	96	32985	Aa	-	10	27	8	2	6	2
N	148	33392	Bb	8	20	24	9	2	7	2
N	191	33923	Bc	8	18	31	9	4	10	4
N	285	34984	Cd	18	1	33	9	1	8	3
N	287	35008	De	-	1	25	9	1	8	3
N	332	35236	De	19	3	25	9	2	10	2
N	335	35247	De	19	3	25	9	2	10	2
N	337	35250	De	19	3	25	9	2	10	2
N	338	35253	De	19	3	25	9	2	10	2
N	341	35261	De	19	3	24	9	2	10	2
N	343	35277	De	19	3	24	9	2	10	2
N	345	35299	De	19	3	24	9	2	10	2
N	350	35320	Fe	19	3	24	9	2	10	2

Patient	Strain	Isol. date	RAPD type *	Hi4-3	Hi4-10	Hi4-11	Hi3-1	Hi5-2	Hi6-1	Hi6-2
V	108	33055	Bf	**26**	15	**39**	9	1	10	3
V	112	33120	Gc	**16**	**21**	**41**	9	6	9	**8**
V	143	33342	Hg	**31**	2	21	9	1	8	**9**
V	227	34575	Ic	16	3	33	9	2	10	3
V	274	34934	Bc	**16**	13	**40**	9	1	9	10
V	291	35010	Jc	**16**	13	**37**	9	1	9	3
V	346	35310	Bc	**22**	3	**21**	-	5	8	3
H	39	32537	Kh	8	15	28	9	2	9	1
H	40	32551	Lh	8	15	28	9	2	9	1
H	49	32756	Mi	21	16	>50	9	7	10	3
H	81	32943	Mi	21	15	>50	9	10	10	3
H	93	32979	Mi	21	15	>50	9	10	10	3
H	111	33118	Mi	21	15	>50	9	10	10	3
H	126	33176	Mi	25	12	>50	9	9	10	3
H	141	33335	Mi	24	12	>50	9	8	10	3
H	146	33378	Mi	25	14	>50	9	11	10	3
H	152	33419	Mi	25	14	>50	9	11	10	3
H	192	33923	Ni	28	3	15	9	4	9	2
H	281	34831	Ni	>40	14	20	9	6	10	7

Patient	Strain	Isol. date	RAPD type *	Hi4-3	Hi4-10	Hi4-11	Hi3-1	Hi5-2	Hi6-1	Hi6-2
G	11	32300	Li	16	3	29	9	1	9	2
G	48	32698	Lj	25	12	25	9	1	10	4
G	68	32894	Lk	25	12	26	9	1	10	4
G	87	32952	Lj	25	11	27	9	1	10	4
G	105	33041	Lj	25	11	26	9	1	10	4
G	305	35138	Lj	-	15	28	9	1	10	4
G	314	35151	--	12	11	28	9	6	0	4
T	72	32911	Xj	6	11	18	9	4	14	6
T	97	32986	Xk	6	2	30	9	1	8	13
T	170	33632	Sl	25	22	30	9	7	8	2
T	221	34430	Xm	11	25	>45	9	2	8	2
T	262	34794	Sm	11	24	>40	9	>40	8	2
T	326	35207	Sm	11	24	>40	9	>40	8	2

Patient	Strain	Isol. date	RAPD type *	Hi4-3	Hi4-10	Hi4-11	Hi3-1	Hi5-2	Hi6-1	Hi6-2
K	42	32554	Li	3	31	32	9	2	10	4
K	79	32924	Xm	2	2	31	9	3	10	4
K	181	33678	Pk	-	1	5	9	6	8	10
K	272	34896	Pj	7	28	16	9	2	9	2
K	300	35101	Qj	8	29	17	9	-	9	2
K	339	35254	Rj	9	2	32	9	2	10	10

NB *RAPD analysis was performed by two PCR assays employing the ERIC1 and ERIC2 primer, respectively. The two letter code describes the interpretation of the separate assays in one combined genocode. >: fragment too large to be accurately sized by agarose gel electrophoresis.
VNTR sizes highlighted in bold were determined by DNA sequence analysis. -, not determined

DNA isolation and PCR mediated typing studies: After overnight growth *H. influenzae* cells were harvested directly into a guanidinium isothiocyanate containing lysis buffer (17). Celite (Janssen Pharmaceuticals, Beerse, Belgium) was added to the resulting lysate and further processing for DNA purification by affinity chromatography took place. The DNA concentration was determined and the solution was stored at a DNA concentration of 10 ng/μl^{-1} at -20°C. Random amplification of polymorphic DNA (RAPD) analyzes were performed as described previously, using a combination of primers ERIC1 and ERIC2 (15). RAPD fragments were analyzed by gel electrophoresis in 2-3% agarose gels (Hispanagar, Sphaero Q, Leiden, the Netherlands) run at 100 mA constant current for 3 h.

Amplification of VNTR regions was done according to recently developed protocol (14). The tetranucleotide VNTR loci Hi 4-3, 4-5, 4-10 and 4-11, present in different potential virulence genes, were analyzed, together with the trinucleotide VNTR Hi 3-1, the pentanucleotide VNTR Hi 5-2 and the two hexanucleotide VNTRs Hi 6-1 and 6-2 (see Table 3 for general description and VNTR codes and PCR primers). Due to the small size of the VNTR amplicons and the required 3-6 nucleotide resolution, these DNA fragments were separated by length on 3% MetaPhor agarose (FMC Bioproducts, Biozym, Landgraaf, the Netherlands). Molecular sizes were determined with the help of 10 basepair ladder (Boehringer-Mannheim, Mannheim, Germany). Gels were run in 0.5xTBE buffer at a constant voltage of 100V. Gels were photographed using a CCD camera equipped with a Fujinon zoom lens. Data were collected and thermo-printed using the Visionary Photo Analyst system (FotoDyne, Progress Control, Waalwijk, the Netherlands).

VNTR cloning and sequencing: For cloning and sequencing, PCR amplified DNA fragments were purified using Qiaquick Column chromatography (Westburg, Leusden, The Netherlands). The amplicons were cloned into pCR1 (Invitrogen, Leek, the Netherlands) according to the manufacturer's instructions. Clones were analyzed with respect to the size of the insert and, when correct, sequenced using cycle sequencing technology and an ABI373 automated sequencer (ABI, Warrington, Great Britain). Sequence data were edited to the appropriate format using 373 Software.

Table 3 Survey of some of the VNTR regions encountered in the genome of *H. influenzae* Rd and monitored for polymorphism in serial isolates from lungs of CF patients.

Repeat until Length	Repeat Code	Repeat Position	Unit Seq	Unit Number	Function	Primers for VNTR amplification	
						5'	3'
3	Hi 3-1	291617- 291644	ATT	9	not known	CAAATGATTATAAATAAACC	TAATTAAAAAGAGGAGAATG
4	Hi 4-3	570800- 570892	CAAT	23	LPS	CCTCTTATATTATGTAAT	TTTAGTTTCTTTAATGCG
4	Hi 4-10	1543152-1543252	TTGC	25	adhesin	GACAGATGAAAAGAAAAGAT	TATAATATGTTTTATTACAA
4	Hi 4-11	1608031-1608099	CAAT	17	LPS	TAAAAATGAATACAAAA	AAGTTTTAACAAATCCTACA
5	Hi 5-2	1368890-1368910	CTCTC	4	not known	GTGATTTTTATCGACAATCT	TACAGAGGGCATAATTTATG
6	Hi 6-1	283097- 283115	CTGGCT	4	not known	TCTACAATTTCTTGTTTTTC	ATGGTGTTGGAAGAACCTGC
6	Hi 6-2	296053- 296071	GGCAAT	3	not known	AGATTTAGAGAGAATCAGTG	CGTCTTTTAGTTTACGGGTA

Note: The columns describing the possible gene function is derived from reference (13); repeat positions are identified on the basis of sequences presented in (12). Primers for the Hi 6-1 repeat are located more distantly from the core units (see section 3).

RESULTS

VNTR sequencing: DNA sequencing for some randomly chosen PCR products (see Table 2, bold figures) was employed to verify the number of repeat motifs as deduced on the basis of the agarose gel electrophoresis and to confirm the sequence of the repeat units. Differently sized VNTR PCR products (Hi 4-3, Hi 4-10, Hi 4-11, Hi 3-1 and Hi 6-2), obtained for the strains isolated from patient V from Rotterdam, were processed. Amplicons obtained with the Hi 6-1 PCR and the Hi 5-2 PCR were not cloned and consequently not analyzed with respect to the precise sequence of the unit motif. All sequence data were in agreement with the consensus unit motifs as described in Table 3. Furthermore, assessment of the number of repeats as present in each amplicon corroborated the figures as deduced from the PCR fragment length determination by agarose gel electrophoresis. VNTR sizes determined by sequence analysis are highlighted in bold in Table 2. During the VNTR analyzes performed for the clinical isolates, sometimes band doublets were observed (see below and Table 1-3). This possibly indicates the existence of multiple alleles for the same VNTR locus in a single strain of *H. influenzae*.

Analysis of MOMP typed strains from CF patients from Amsterdam: MOMP diverse strains shown VNTR length variability in all loci monitored except for Hi 3-1 and Hi 6-2 (see Table 1). Variation in Hi 5-2 is only documented for patient CF32. Altogether, two sets of strains having identical MOMP characteristics were studied. For patient CF30 it can be seen in Table 1 that variation occurs in two out of three tetranucleotide VNTRs assayed and Hi 3-1, Hi 5-2, Hi 6-1 and Hi 6-2 do not seem to be variable. For the strains from patient CF32 variability was documented for Hi 5-2 and 6-1. In conclusion, all of the VNTRs show polymorphism, despite the fact that they may be present in MOMP- and RAPD-identical strains. Some of the VNTRs differ in one patient, but remain unaltered in the other, showing dependence on the individual that is colonized. Among strains isolated from a single patient the variability in size is generally smaller than the variability seen upon analysis of strains derived from different patients.

Analysis of serially isolated strains derived from patients from Rotterdam: Among these strains again Hi 3-1 was present as a relatively stable genetic marker: in nearly all cases (except one) the number of repeat units is nine (see Table 2). Furthermore, if overall genetic diversity

is determined by RAPD, associated variability among the VNTRs is encountered. Among these strains even Hi 6-2 presents as a genuine VNTR (see data obtained for strains from patient K). An more important feature of this collection of strains is the fact that serial isolates, sometimes spanning many years, are included. This is documented by identical RAPD identification codes (Table 2). For patients V, T and G paired strains of identical genotype but different isolation data are available. In these cases clear polymorphism in various VNTRs have been documented. On the other hand it is interesting to note that other situations can also be observed: in patient T two strains harbor the RAPD type Sm (numbers 262 and 326). Here the VNTRs have remained unchanged. For patient H nine strains with RAPD type Mi were available. Multiple changes in VNTRs Hi 4-3, Hi 4-10 and Hi 5-2 became apparent. The other VNTRs were stable during the entire screening period. For patient N eight strains sharing genotype De were retrieved. Only the first isolate (strain 287) differed with respect to the VNTR composition. However, this isolate was cultured one year prior to the other ones, which were collected upon different occasions during a two months period. Moreover, for patient H strains 192 and 181 share an RAPD pattern Ni, whereas the VNTRs are clearly different. In conclusion, various degrees of VNTR size variability can be documented by PCR when a given patient is colonized for prolonged periods of time by a single genotype of *H. influenzae*. The VNTRs Hi 3-1, Hi 6-1 and Hi 6-2 are relatively constant in size, although incidental differences can be determined. Most VNTRs assayed here appear to be variable, independent of the stable genetic background that they are part of.

DISCUSSION

Various repeat regions built from di- to hexanucleotides were identified in the full genome sequence for the *H. influenzae* Rd strain (12, 14, 18). For all of the 3-6 nucleotide repeats in the *H. influenzae* chromosome, specific PCR test capable of detecting allelic polymorphism were designed (14). The length of the VNTRs was a stable genetic marker for separate colonies derived from a single clinical specimen or strains passaged in the microbiology laboratory for several weeks on chocolate agar plates. When several strains isolated from different patients during an outbreak of lung disease caused by *H. influenzae* were analyzed (15), increased but limited variation was encountered in the four-nucleotide unit VNTR sites (19). It appeared that the tri-, penta- or hexanucleotide VNTRs were more stable in nature and consequently more suited for epidemiological studies. One of the two five-nucleotide VNTRs, however, proved to be hypervariable when strains isolated during a local outbreak of *H. influenzae* infections were studied (14).

All tetranucleotide VNTRs appeared to be associated with bacterial virulence genes and molecular knock-out of one of these genes resulted in attenuated virulence (13). The nature of these genes varied from the well-known LPS biosynthesis genes, adhesin and glycosyltransferase encoding genes to several iron binding protein genes. It is demonstrated that during persistent colonization of CF lung tissue by genetically homogeneous clones of *H. influenzae*, serially occurring alterations in VNTR lay-out can be observed. These changes do affect the coding potential of the genes in which the repeats are located: frequently the variable numbers of repeats encountered cover all three theoretical reading frames. Apparently, different waves of sub-clones of *H. influenzae* develop, where these sub-clones share an identical whole genome organization but differ with respect to the composition of their virulence gene repertoire. It should be realized that an entire population of bacterial cells is replaced by a 'new' one, most probably as a result of the subtle interplay between bacterial contingency behavior and human defense mechanisms. It is interesting to note that besides the repeat-associated variability, *H. influenzae* strains that persistently colonize the lungs of CF patients can also vary metabolic enzyme activities. It has been documented that changes in biotypes in genetically constant strains occur with relative ease and irrespective of variation occurring in the major outer membrane protein (12). It has to be emphasized, however, that final

conclusions can only be drawn once these latter studies have been complemented with somewhat more detailed DNA sequencing studies.

The general conclusion of the present data is that during persistent colonization of CF tissue *H. influenzae* displays complicated population dynamics. Strains can be replaced by different genotypes (see also (5) for instance), but also within the genome of a single, persisting strain structural changes leading to adaptation of virulence factor expression can occur. It is this kind of contingency behavior that may facilitate subclones to replace ancestral strains. The micro-environment within the CF lung may facilitate selection of this type of subclones and predispose the patient to those strains that are optimally adapted to the CF niche. The notion that many bacterial species may be capable of employing repeats for adaptation of their virulence gene potential is a challenging one (20). This should stimulate additional clinical studies to be undertaken.

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Chapter 7

Comparison of conventional and molecular methods for the detection of bacterial pathogens in sputum samples from cystic fibrosis patients

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ABSTRACT

The nature of the micro-flora present in sputa of six different cystic fibrosis (CF) patients was assessed using routine microbiological culture and molecular methods. Bacterial genes for the small sub-unit ribosomal RNA (ssu rDNA) were specifically amplified from DNA extracted from the sputum samples, cloned and characterized by hybridization and DNA sequencing. A large number of clones from 6 sputa were screened. Initially, oligonucleotide hybridization was performed with five probes, specific for Gram-positives and Gram-negatives in general and the main pathogens for the CF patient (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*). For a single sputum sample, the results were fully congruent when culture and molecular methods were compared. In the other five sputa, discrepancies for *S. aureus* and/or *H. influenzae* were documented. Although *S. aureus* DNA and *H. influenzae* DNA was detected in three and four sputa, respectively, strains could not be cultured. Although the PCR approach is not capable of distinguishing viable from dead bacteria, all of the CF patients had a history of *S. aureus* infections, while one of the CF patients once had cultivable *H. influenzae* in the sputum as well. A number of clones for probe-unidentified Gram-negative or Gram-positive bacterial species were further analyzed by sequencing and additional potential pathogens were identified. Although routine culture of sputum frequently points to mono-specific exacerbations, our molecular data indicate that the other CF-related pathogens appear to be persistently present as well. We conclude that routine culture for bacterial pathogens from CF sputa yields limited microbiological information since it frequently fails to identify a number of pathogenic bacterial species that are potentially present in a viable status in the lungs of these patients.

INTRODUCTION

Cystic Fibrosis (CF) is the most common autosomal recessive genetic disorder in North Europeans. The major clinical problem of CF patients is the progressive loss of pulmonary function, usually due to bacterial infections that contribute to an ultimately fatal lung disease. *Staphylococcus aureus* and *Haemophilus influenzae* are the most common bacterial pathogens early in life, *Pseudomonas aeruginosa* is usually encountered in older CF patients. Previous longitudinal studies showed that most CF patients had periods of long-term colonization by one predominant *S. aureus* genotype, but variation in *S. aureus* genotypes within single patients was also observed (1). When the CF patients became colonized with *P. aeruginosa*, the same results were seen, namely long-term colonization by one or two genotypes (2). If the CF patient was colonized with *H. influenzae*, different strains were isolated from a single sputum specimen and a predominant strain persisted over time (3). Initially, CF patients are colonized with non-mucoid *P. aeruginosa* strain. After a while, the *P. aeruginosa* strains start producing an extra-cellular alginate polysaccharide and their colonies on agar become mucoid. The presence of mucoid *P. aeruginosa* may obscure recognition of *S. aureus*. Moreover, it also has been documented that *P. aeruginosa* is capable of producing substances that inhibit the growth of *S. aureus* (4). So far, there are no pseudomonal compounds identified that impair the growth of *H. influenzae*. These observations substantiate the notion that bacterial colonization of the CF lung is a dynamic process. Additional investigations into the interactions between patient and bacteria and between different species of bacteria are still required.

In order to analyze the bacterial population dynamics and complexity in the CF lung, improvements in microbiological diagnostics are mandatory. Shortcomings of the current, straightforward microbiological sputum analysis have been suggested in the past and efforts to improve the methodology have been documented in the literature (5, 6). However, none of the present procedures can guarantee exquisite sensitivity due to features like bacterial interference or the hardly detectable presence of metabolically and physiologically altered bacterial cells such as the small colony variant of *S. aureus* (7). DNA-based diagnostics can circumvent these problems simply, due to the fact that DNA isolation from sputum samples is experimentally simple and is not negatively affected by bacterial viability, competition for

nutrients or the presence of growth inhibitory compounds. Various strategies have been described for assessing the presence of mixed bacterial populations of unknown species in different ecological niches. The PCR-based amplification of ribosomal genes, for instance, has revealed bacterial population complexity in various clinical syndromes such as dento-alveolar abscesses in adults (8) or necrotising enterocolitis in preterm infants (9). It was also used for detection of bacterial DNA in the middle ear effusions of children suffering from otitis media (10). During the present study we have applied PCR amplification of a fragment of the bacterial small subunit ribosomal DNA (ssu rDNA), random cloning of amplified DNA and probe-mediated or DNA sequencing-based identification of individual clones for assessment of bacterial population heterogeneity in sputa obtained from CF patients. The aim of this study is to determine the presence of the major pathogenic species *H. influenzae, S. aureus* and *P. aeruginosa* in long-term colonized CF patients with molecular methods and to compare the results with standard microbiological culture.

MATERIALS AND METHODS

Patients and clinical specimens: Sputum samples were obtained from six patients with CF in October 1996 in the Erasmus University Medical Center Rotterdam (The Netherlands). The nature of the antibiotics used by the CF patients at the time of collection is mentioned in Table 2. The age of the patients ranged from 21 to 32 years. All of the patients were taking part in the CF surveillance system that is applied in our hospital. Although the same culture procedures were not always used over the years, with the use of the available strains and clinical data, the longitudinal colonization pattern of these CF patients could be assessed from the beginning of the colonization until October 1996. The period of surveillance varied from 4 up to 11 years. Patient 1 was colonized with *S. aureus* since 1990 and *P. aeruginosa* was isolated sporadically since. Patients 2 and 5 were persistently colonized with *P. aeruginosa* and *S. aureus* was isolated once in 1988 and in June 1996, resp. Patient 3 was colonized with *S. aureus* and *P. aeruginosa* since the end of 1992. Patients 4 and 6 were initially colonized with *S. aureus* and after 5 and 4 years, respectively, they became colonized with *P. aeruginosa* as well. *H. influenzae* was isolated only from patient 1, sporadically in 1991 and 1992.

Microbiological culture techniques: Portions of sputum were washed three times in physiologic saline solutions. A small portion of sputum was transferred to petri dishes containing saline and shaken for approximately 1 min each. Without prior mucolytic treatment, the sputum sample was split in half, one portion was used for DNA isolation (see below) and the other half was used for routine culture. Gram-staining was used to quantify leucocytes, erythrocytes and bacterial content of the sputa (see Table 2). Sputa were inoculated on Columbia agar for detection of Gram-positive cocci, on MacConkey agar (37°C with CO_2 for 24 h and without CO_2 another 24 h) for Gram-negative rods and on chocolate agar without bacitracin (37°C with CO_2 for 48 h) for *H. influenzae*. Phenol mannitol salt agar (37°C with CO_2 for 24 h and without CO_2 another 24 h) was employed for the detection of *S. aureus*. All growth media were purchased from Becton Dickinson (Heidelberg, Germany). Different colonies growing on the various media were identified by standard microbiological testing (11).

DNA isolation: DNA was isolated from CF sputum samples (5-10 mg) through a protocol that started with an initial lysis procedure employing SDS and proteinase K (12). After 30 minutes of incubation at 37°C all biological material was lysed by addition of a buffer containing the chaotropic agent guanidinium isothiocyanate and the detergent Triton X-100. DNA was subsequently purified by affinity chromatography on Celite (Janssen Pharmaceuticals, Beerse, Belgium). The DNA concentration in the stock solutions was determined by agarose electrophoresis where samples of the DNA were run in parallel with known amounts of bacteriophage lambda DNA that served as references. Stocks were stored at –20°C.

PCR-mediated DNA amplification and cloning: Amplification of ssu rDNA was performed according to Greisen et al (13) and by use of the universal primers DG74 and RDR080 (5'-AGGAGGTGATCCAACCGCA-3' and 5'-AACTGGAGGAGGAAGGTGGGAC-3', respectively). The location of these primers on the ribosomal consensus sequence is defined by positions 1522-1540 and 1170-1189, respectively. Consequently, an amplimer of approximately 350 bp in length was synthesized. The amplimers thus obtained were randomly cloned into the plasmid pCR1 using the original TA cloning kit version E (Invitrogen, Leek, The Netherlands) (14). After ligation, the recombinant plasmids were transformed into

competent *Escherichia coli* JM109 cells. For each sputum sample, a variable number of ampicillin-resistant transformants, further identified by a white color on LB agar containing IPTG and X-gal, were selected for analysis.

Molecular identification of ssu rDNA clones: Individual clones were transferred to fresh LB agar containing ampicillin and after overnight growth at 37°C single colony lifts were prepared onto Hybond N^+ filters. The resulting dot blots were initially analyzed by specific hybridization with radioactively labeled oligonucleotide probes recognizing ribosomal clones from either Gram-negative or Gram-positive bacteria (5'-GACGTAAGGGCCATGACTTGACGTC-3' and 5'-GACGTAAATCATCATGCCCCTTATGTC-3', respectively (13). The probes were labeled at the 5' terminus using T4 kinase (Boehringer-Mannheim, Mannheim, Germany) and γ-^{32}P labeled ATP (Amersham, Buckinghamshire, England). Unincorporated nucleotides were removed by gel filtration using spin columns. Hybridization was performed at 50°C at high stringency. Clones that did not hybridize with one of the Gram-probes or hybridized with both were excluded from further evaluation. Subsequently, the blots were hybridized to *H. influenzae, S. aureus* and *P. aeruginosa* specific DNA oligonucleotide probes (5'-GGAGTGGGTTGTACCAGAATAGAT-3', 5'-GCCGGTGGAGTAACCTTTTAGGGAC-3' and 5'-CGTGAATCAGAATGTCA-3', respectively (13). The *P. aeruginosa* probe was newly developed on the basis of sequence comparisons among related species (see Table 1 for comparison and probe sequence). In order to enhance the specificity of the *H. influenzae* probe, which initially cross-hybridized to *P. aeruginosa* clones as well, we had to modify its sequence to 5'-TACCAGAATAGATAGCT-3'. This probe did not show any cross-hybridization to the *P. aeruginosa* clones (unpublished data). Assessing the precise specificity of the probe has not been pursued because of the assumption that the specificity would be similar to that of the probe described by Greisen et al (13). The probe did not detect homologues in other sequences available in the GenBank database. In order to confirm the nature of the clones thus identified, several of them were sequenced using the Prism Ready Reaction Dye Di-deoxy terminator cycle sequencing kit (Amersham Int plc, Gouda, The Netherlands). Plasmids used as templates for the sequencing were purified according to Birnboim (15). In addition, several clones positively identified as containing an insert hybridizing with either the Gram-positive or the Gram-negative confirmation probe and none of the species-specific oligonucleotide probes

were sequenced as well. Sequencing products were analyzed on an ABI373 DNA sequencer using the software as supplied by the manufacturer (ABI, Gouda, The Netherlands). For sequence comparisons and identification of ribosomal motifs, BLAST searches were performed against the nucleotide sequence data available from various databases (16, 17).

Table 1 Comparative analysis of the rRNA region for various species including *Pseudomonas aeruginosa*: development of a novel molecular probe for species-specific identification of *P. aeruginosa*

Species	rDNA sequence				
	|1341	|1351	|1361	|1371	|1381
Pseudomonas aeruginosa	GTCGGAATCG	**CTAGTAATCG**	**TGAATCAGAA**	**TGTCACGGTG**	AATACGTTCC
Pseudomonas fluorescens	GTCGGAATCG	CTAGTAATCG	CGAATCAGAA	TGTCGCGGTG	AATACGTTCC
Acinetobacter baumanii	GTCGGAATCG	CTAGTAATCG	CGGATCAGAA	TGCCGCGGTG	AATACGTTCC
Bordetella bronchoseptica	GTCGGAATCG	CTAGTAATCG	CGGATCAGCA	TGTCGCGGTG	AATACGTTCC
Burkholderia cepacia	GCTGGAATCG	CTAGTAATCG	CGGATCAGCA	TGCCGCGGTG	AATACGTTCC
Neisseria gonorrhoeae	GTCGGAATCG	CTAGTAATCG	CAGGTCAGCA	TACTGCGGTG	AATACGTTCC
Acinetobacter lwoffii	GTCGGAATCG	CTAGTAATCG	CGGATCAGAA	TGCCGCGGTG	AATACGTTCC

Note: The actual probe sequence is presented in bold, mismatches in the probe target region for the other organisms are highlighted by underlining as well. Numbering indicates the position in the 16S rRNA gene

Table 2 Results of the determination of the microflora of 6 CF sputa by cultural and molecular methods.

Molecular analysis					Routine microbiology			
Patient number	Gram probe	Species[a]	Number	Number of evaluable clones	Gram-stain	Gram-quant.	Culture	Antibiotic treatment at time of collection
1	+	*S. aureus*	80	313 (61%)	Leucocytes	2	*S. aureus*	Flucloxacillin
	+	?[b]	4		Gram-positive cocci	3		
	-	*H. influenzae*	127					
	-	*P. aeruginosa*	1					
	-	?[b]	101					
	+	*S. aureus*	138					
2	+	*S. aureus*	138	624 (64%)	Leucocytes	1	*Pseudomonas* species, 2x	Sulfamethoxazole+
	+	?[b]	75		Gram-negative rods	2		trimethoprim
	-	*H. influenzae*	82					
	-	*P. aeruginosa*	195					
	-	?[b]	134					
3	+	*S. aureus*	103	536 (62%)	Leucocytes	3	*S. aureus*	Sulfamethoxazole+
	+	?[b]	69		Gram-positive cocci	4	*Pseudomonas* species, 2x	trimethoprim
	-	*H. influenzae*	0		Gram-negative rods	4		
	-	*P. aeruginosa*	338					
	-	?[b]	26					

Molecular analysis					Routine microbiology			
Patient number	Gram probe	Species[a]	Number	Number of evaluable clones	Gram-stain	Gram-quant.	Culture	Antibiotic treatment at time of collection
4	+	*S. aureus*	100	220 (28%)	Leucocytes	2	*Pseudomonas* species	Ciprofloxacin
	+	?[b]	98		Epithelial cells	1		Colistine spray
	-	*H. influenzae*	3		Gram-positive cocci	2		
	-	*P. aeruginosa*	4		Gram-negative rods	2		
	-	?[b]	15					
5	+	*S. aureus*	0	260 (26%)	Leucocytes	2	*Pseudomonas* species, 3x	Meropenem
	+	?[b]	0		Erythrocytes	4		Tobramycin
	-	*H. influenzae*	73		Gram-negative rods	1		Clistine spray
	-	*P. aeruginosa*	0					
	-	?[b]	187					
6	+	*S. aureus*	9	605 (59%)	Leucocytes	3	*Pseudomonas species*, 2x	Ciprofloxacin
	+	?[b]	5		Gram-negative rods	2		Ceftazidime
	-	*H. influenzae*	0					Colistine spray
	-	*P. aeruginosa*	451					
	-	?[b]	140					

Note: The column "Gram quant" states the Gram-derived cellular quantities: 1 = 1 cell per field; 2 = 2-10 cells per field; 3 = more than 10 cells per field; 4 = more than 100 cells per field. One field is a single microscopic field at 1000x magnification. 2X or 3X in the column indicating the culture results means that 2 or 3 different species could be discriminated on the basis of difference in colony morphology. [a]: identification with oligohybridization; [b]: no identification with one of the species specific probes.

RESULTS

A total number of 5164 clones were evaluated, 2558 of which hybridized correctly with the Gram-probes. The results from the analysis of the sputum samples are shown in Table 2. Molecular analysis, using the *S. aureus* or *P. aeruginosa* or *H. influenzae* probes, identified 1704 clones to the species level (see Figure 1 for an example of the experimental output). It is interesting to note that among the clones from the sputum samples, relatively large numbers of unidentified recombinants (both Gram-positive and Gram-negative) were still present (n=854). A positive signal with the Gram-negative probe and no signal with the *P. aeruginosa* or *H. influenzae* probe was found for 603 clones. A number of these unidentified clones were selected at random and sequenced. Seven clones were identified as *H. influenzae*, 13 as *Escherichia coli*, two as *Klebsiella pneumoniae*, four as *Pseudomonas citronellosis*, two as *P. aeruginosa* and in two instances, a homology with human artefact sequences was documented. A total of 251 clones gave a positive signal with the Gram-positive probe and no signal with the *S. aureus* probe. Fourteen of these unidentified clones were sequenced, 13 clones were identified as *S. aureus* and one as *Streptococcus macedonicus*. The fact that 13 out of 14 clones represented *S. aureus* DNA shows a weakness of the hybridization procedure that was used for clone identification. The fact that clones belonging to the species that we were specifically searching for remained unidentified, but could be due to smaller numbers of cells in the colony lifts. Upon re-examination of the blots, it appeared that the signals obtained with the Gram-probes were also weak in these instances (results not shown). Several of the clones that were correctly identified by the combined application of the oligonucleotide probes were re-evaluated by DNA sequencing. All *S. aureus* (n=6), *P. aeruginosa* (n=10) and *H. influenzae* (n=3) probe-reactive clones displayed correct sequences.

S. aureus was cultured from sputa 1 and 3. With the molecular method rDNA of *S. aureus* was not only found in sputa 1 and 3 but also in sputa 2, 4 and 6. *H. influenzae* was cultured in none of the 6 sputa, but rDNA of this species was identified in sputa 1, 2, 4 and 5. *Pseudomonas* spp were cultured from sputa 2, 3, 4, 5 and 6. The strains were all mucoid and were not identified to the species level. In all the sputa, rDNA of *P. aeruginosa* was found, except in sputum 5. Probably, this patient was colonized with a *Pseudomonas* non-aeruginosa species. In patient 1, a single clone of *P. aeruginosa* was identified. The meaning of this

finding is unknown, but in 1997, this patient became colonized with *P. aeruginosa* and *S. aureus* was isolated less frequently. It must be emphasized that many more species may be hidden among the clones that were not studied in detail.

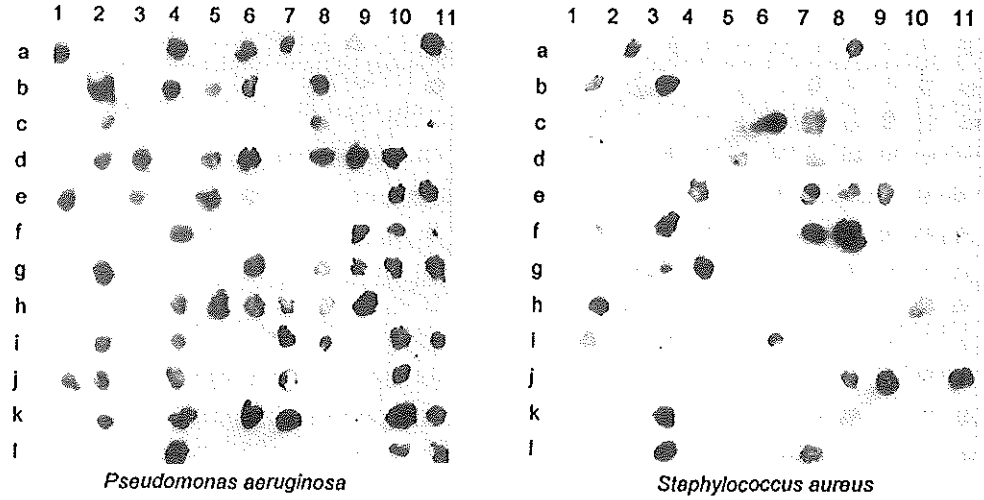

Figure 1: Dot blot assay for discrimination of *Pseudomonas aeruginosa* and *Staphylococcus aureus* clones on the basis of oligonucleotide hybridization. Colony lifts were prepared from 132 Independent bacterial clones and the resulting blot was hybridized with a probe specific for *P.aeruginosa* rDNA (panel on the left) or S. aureus rDNA (panel on the right). The dot on position 1a in the panel on the left identifies a *P. aeruginosa* recombinant, the one on position 2a in the right panel identifies a *S. aureus* clone. At position 1k for instance neither panel shows positive hybridization, which is indicative of the presence of an insert different from the ones for the two species mentioned above.

DISCUSSION

In the present study, we demonstrate that routine microbiological cultivation assays performed on sputum samples from CF patients do not suffice and frequently do not even fully concur with simple tests such as Gram-staining (see Table 2). For instance in the case of sputum 4, between 2-10 Gram-positive cocci were observed per microscopic field. The presence of staphylococci is corroborated by the molecular study but culture did not give rise to growth of *S. aureus*. Also, as can be seen from the quantitative Gram-staining data, there is no apparent concordance between the lack of positive cultures and the amount of cells as identified on the basis of the Gram-staining and subsequent microscopic analyzes. In all cases, except for sputum 5, only one or two bacterial species were documented by culture in the clinical microbiology laboratory.

Our study demonstrates for the first time the universal presence of multiple potentially pathogenic bacterial species in the sputum of CF patients. This observation argues for changes in the diagnostic protocol. It has to be emphasized, however, that our analysis might be blurred by the fact that the CF patients have to follow a nearly continuous regimen of antibiotic consumption. Since the PCR does not distinguish between living and dead organisms, the comparison with culture may not be completely fair. In defense of our approach it is demonstrated for sputum 1, for instance, that the flucloxacillin therapy, aimed at *S. aureus* did not prohibit a positive culture result. In the other sputa, viable organisms were also detected, despite use of adequate antibiotics as determined by culture results. These latter observations, together with the fact that long-term persistence of bacterial DNA in the CF lung is deemed unlikely, due to high levels of sputum production, suggest that we are detecting rDNA from viable and, consequently, potentially pathogenic bacterial populations. Although all patients received anti-microbial prophylaxis at the time of sample collection, some interesting observations can be made with respect to bacterial viability. The therapy applied with patient 1 for instance, should not affect Gram-negative bacteria, whereas the use of colistine spray should not act against *S. aureus* (see Table 2, patients 4 and 6). Thus, we have to conclude that not all patients received effective broad spectrum therapy, which argues for the fact that the microorganisms detected by the molecular approach were likely to be alive at the moment of harvesting the clinical material.

The present data suggest that microbial diagnostics in CF patients may only be useful for assessing the nature of the pathogen causing an exacerbation. Previous studies showed that most of the CF patients remain colonized for a long period with an identical genotype of *S. aureus* and / or *P. aeruginosa* (1, 2). This suggests that once a patient is colonized with a given microbial species, it will most probably persist, despite the use of anti-microbial therapy (18) and even though culture yields negative results. This is also a distinct possibility for those bacterial species that could be identified by sequence analysis of cloned rDNA fragments. For instance in sputum 1, *E. coli*, *K. pneumoniae* and *H. influenzae* could be identified on the basis of sequence homology searches and additional research on the putative persistency of these bacterial species is certainly indicated. In the past, several cases describing similar phenomena have been reported. Whitby et al, for instance, detected *Burkholderia cepacia* DNA in culture-negative CF sputa with a species-specific PCR. These patients were previously demonstrated by culture to be intermittently colonized (19).

The efficiency of PCR, cloning and hybridization with species-specific oligonucleotide probes for detection of *S. aureus*, *H. influenzae* and *P. aeruginosa* was analyzed and compared to the results of microbiological culture. This demonstrated that the technically more demanding molecular procedure appeared to be more sensitive than cultivation. In five *Pseudomonas spp.* culture-positive sputa, *S. aureus* failed to grow in four sputa and *H. influenzae* in three. Patient 2, for instance, had never been diagnosed with *H. influenzae,* but only with a *Pseudomonas spp.* infection in the period of 10 years that she was regularly visiting our hospital for check-ups. In the molecular diagnostics analysis, however, 83 out of 411 Gram-negative clones that were specified represented *H. influenzae.* In this group of patients, specific therapy aimed at a single microbial species may provide an excellent opportunity for one of the other species that are sub-clinically present to fill the ecological niche that becomes vacant upon such an occasion. True quantitative interpretation of the data that we provide is not feasible since the ratio observed between different species present in the sputa may well be different from the ratio observed between species on the basis of numbers of clones. It may be that a given template amplifies to a lesser (or a larger) extent. Also the numbers of rRNA genes per genome differ between species. If the PCR data are to be useful clinically in the future, then quantification of each species within a sputum sample will be a requirement.

In conclusion, it can be stated that microbial diagnostics and therapies that are based on culture only are inadequate. Alternatives or suggestions for improvement of microbiological and clinical care are, however sadly, hard to provide at this stage. Firstly, the molecular procedure described in the present communication is far too complicated to be implemented in routine diagnostics. Secondly, research on the viability of the bacterial species identified by rDNA PCR only should be performed. Last but not least, further molecular analyzes of the micro-flora present in the lungs of CF patients in relation to the stages of their disease and therapies provided are needed to elucidate the role of micro-organisms in the pathogenesis and prognosis of CF.

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Chapter 8

General discussion

GENERAL DISCUSSION

Microbial typing studies are needed once it is suspected that a series of isolates from various sources are epidemiologically linked or that multiple isolates of the same species isolated during the course of an infection in a single patient are clonally related. The level of clonal relatedness is dependent on the nature and number of characteristics that are shared by different strains of a given microbial species. Typing systems are based on the premise that clonally related isolates will share characteristics by which they can be differentiated from unrelated isolates. The utility of a particular characteristic for typing is related to its stability within a strain and its diversity within the species. Several criteria are useful in evaluating typing systems: typeability, reproducibility, discriminatory power, ease of interpretation and feasibility.

For our study we compared phenotyping techniques and genotyping techniques mainly for *Pseudomonas aeruginosa*.

Classical strain typing techniques are based on the presence or absence of metabolic or biologic activities. Such phenotyping techniques, including antimicrobial susceptibility profiling, pyocin sensitivity or pyocin production testing, susceptibility to phages, biotyping and O-serotyping have been used for epidemiological investigations of *P. aeruginosa*. Each of these methods has its advantages and disadvantages.

Phenotyping of *P. aeruginosa* strains from CF patients is difficult, because differing antibiograms can be observed in strains cultured from a single sputum sample of an individual patient and antibiograms may change under the selective pressure of antimicrobial therapy. Pyocin types are also subject to change in response to antimicrobials. It is known that a biotype can change during the course of an outbreak. Bacteriophage sensitivity testing of morphological different strains, mucoid and non-mucoid, may yield different sensitivity patterns. Serotyping is the most commonly applied method of typing isolates from CF patients, however, up to 70% of the isolates may either be non typeable or agglutinate in multiple O-typing sera. Each of these methods have been effective for typing in certain clinical settings, but most have also been found inadequate under conditions in which *P. aeruginosa* undergoes spontaneous phenotypic conversion.

The more recently developed molecular typing techniques such as restriction fragment

length, polymorphisms of bacterial genes, PCR based on analysis of (parts of) genomes and nucleotide sequence analysis, have proved to be powerful tools for differentiating isolates.

In chapter 2 we demonstrate the lower resolution of sero- and pyocin typing procedures when compared to DNA typing by PFGE and AP-PCR. PFGE is a variation of agarose gel electrophoresis in which the orientation of the electric field across the gel is changed periodically. This enables DNA fragments as large as megabases to be separated effectively on the basis of their size.

AP-PCR, also referred to as RAPD, is based on the use of short primers whose sequence is not directed to any known genetic locus but will nevertheless randomly hybridize chromosomal sites with sufficient affinity to permit the initiation of DNA polymerization. If two sites are located within a few kilobases of each other on opposite DNA strands then amplification of the intervening fragment will occur.

As stated above the phenotypic procedures, used in chapter 2, displayed lower typeability and discriminatory power than PFGE and AP-PCR; the latter two generally showing concordance. A reason for the small discrepancies observed may be the lack of standardization of the rules applied for the description of (sub)clonal relationships based on genotyping results. In the literature, the definition for a PFGE subclone is based on positional differences in the position of up to five bands from the index type, whereas in case of AP-PCR a single band difference led to the definition of another type. These widely differing approaches for clone/subclone description may explain most of the differences encountered between PFGE and AP-PCR data as shown in chapter 2.

Chapter 3 focuses on determination of subclonal variants *P. aeruginosa* strains based on AP-PCR patterns in comparison with PFGE patterns. Typing systems such as PFGE of DNA macrorestriction fragments and AP-PCR analysis have been used for detailed comparisons among clinical and environmental strains of *P. aeruginosa*. However, only a few studies have been published in which the efficacies of the typing strategies were compared. The relationship between data obtained by PFGE and AP-PCR for the same collection of *P. aeruginosa* isolates was discussed. These authors concluded that AP-PCR can serve as a first screen for *P. aeruginosa* typing for two reasons: it is rapid and it is relatively easy to apply.

Our study as described in chapter 3 indicates that if single band differences between AP-PCR derived fingerprints are ignored, there is excellent agreement between AP-PCR and the

PFGE-based grouping of *P. aeruginosa* strains. Unfortunaly, both techniques have their limitations. PFGE has two limitations, the preparation of suitable DNA can vary in duration from 2 to 4 days because of the need to diffuse all buffers and enzymes into the agarose insert and it requires expensive, specialized equipment. Another problem is the selection of the restriction enzymes. The primary selection criterion for restriction enzymes used for PFGE pretreatment should be the presence of a sufficient number of restriction sites to allow adequate discrimination and resolution. AP-PCR has problems in achieving reproducible, discriminatory results because the reaction conditions during PCR are less stringent; stringency is reduced in order to facilitate the initiation of the polymerization reactions at sites having one or more mismatches. Moreover, the quantities of template DNA may vary among the different fragments amplified from an isolate.

In case of *P. aeruginosa*, we found that the existence of as many as six differences between the PFGE generated DNA banding patterns may not rule out clonal relatedness. The AP-PCR data obtained with primer ERIC2 were interpreted in a straight forward manner: only single band differences should be ignored. This would allow the following typing scheme: by screening with AP-PCR clonal relatedness can be determined rapidly, easily and at relatively low costs. In a second stage, PFGE could be used for confirmation of the AP-PCR data and for the definition of a so called library type. Library types are final and useful for establishing databases amenable to expansion with novel types in the future. Library typing allows for detailed retrospective analyzes. However, no such definite system is yet available for *P. aeruginosa*, *H. influenzae* or *S. aureus*, although our studies certainly allow for adequate evaluation of the procedures that are currently available. Future studies using multilocus sequence typing or DNA chip technology will reveal the feasibility of developing library typing systems for the three CF pathogens of primary importance.

In the next three chapters we determined colonization patterns for the three primary CF pathogens: *H. influenzae*, *S. aureus* and *P. aeruginosa*.

In chapter 4 we present a family-band study of *P. aeruginosa* colonization in the lung of CF patients in order to further elucidate the long-term colonization and spread of *P. aeruginosa* strains within the family milieu.The diversity and variability of *P. aeruginosa* strains in long-term colonized CF siblings were investigated with molecular typing using a single AP-PCR assay.

Høiby considered a patient to be chronically colonized if cultures of sputum samples yielded *P. aeruginosa* for six consecutive months and/or when two or more precipitins against *P. aeruginosa* were present in the serum. However, our study showed that detailed genetic analysis of the *P. aeruginosa* isolates is needed before concluding that an infection is chronic, because clones frequently are lost and required, especially in the beginning of the phase of infection with *P. aeruginosa*. It seems to be that before the lungs become persistently colonized with one or two stable genotypes, infections with several clones of *P. aeruginosa* will take place.

Several studies have reported on the patterns of chronic infection, including studies on colonization with a single strain, periodical replacement of a predominant strain or colonization with multiple, coexistent strains. Cross-infection has been documented frequently. Some of these discordant results may be due to methodological differences, including duration of follow-up and the typing method used. In our study most patients were persistently colonized with a single genotype of *P. aeruginosa*, and only a few patients were colonized with more than one genotype of *P. aeruginosa*. Cycles of carriage, with loss of strains and appearance of new strains were observed. Interestingly, identical genotypes were only found when strains isolated from CF patients in a family were compared. Between families, no overlap in colonizing strains was encountered. Previous studies have shown that siblings can be colonized with identical genotypes of *P. aeruginosa*. However, in our study only two out of six pairs of siblings acquired a genotypically identical long-term colonizing strain. This observation supports the theory that short-term cross-contamination between related patients may occur, but that such cross-contamination only rarely leads to persistence of the cross-contaminating strain. If cross-infection was the most important origin of infection in the family situation, we would have expected to find a higher colonization rate with identical strains in our pairs of siblings. The results of our study also showed that the younger sibling did not become colonized or infected with *P. aeruginosa* in the same period as the elder one. If prolonged intimate contact is needed for cross-infection than we would have expected that siblings should become infected at more or less the same time. A number of reports have been published showing that a higher percentage of CF patients treated at a special CF Center acquired *P. aeruginosa* earlier than those treated elsewhere. After separation of non-infected from infected patients and implementing simple hygienic precautions, a decrease in the yearly incidence of new *P.*

aeruginosa infection among CF patients was noted. These results are in apparent contradiction when compared with the sibling studies. Possibly the clinical state of the respiratory tract and the receptiveness to particular strains within the CF lung's environment are more significant factors for establishing long-term colonization than proximity alone. Further research about the clinical relevance of cross-infection needs to be done.

Chapter 5 describes our study on the epidemiology of airway colonization with *S. aureus* in CF patients. The persistence of *S. aureus* in young CF patients is a well known phenomenon but the epidemiology has not been studied extensively. In our study most CF patients exhibited periods of long-term colonization by one predominant *S. aureus* strain and in a few patients cycles with several *S. aureus* clones were observed. Replacement of a predominant clone by another was also seen. Identical *S. aureus* genotypes were found in unrelated CF patients as well as in the siblings. We observed the ability of *S. aureus* to persist and interchange in this patient population. A remarkable feature is that most of the younger CF siblings become colonized earlier with *S. aureus* than the older ones. The variation in *P. aeruginosa* genotypes is much higher than in *S. aureus* genotypes, indicating that *S. aureus* as a species is more clonally organized than *P. aeruginosa*. One genotype of *S. aureus* was isolated in 33% of the CF patients and accounted for 20% of all tested strains. This is in contrast to what we found in our *P. aeruginosa* study (chapter 4). Additional search for factors that can play a role in bacterial colonization is needed to demonstrate if this genotype is typically associated with cystic fibrosis.

In chapter 6 we investigated the variation in so-called tandem repeat elements in the genome of *H. influenzae* isolated from CF patients. Various repeat regions consisting of di- to hexanucleotides are identified in the full genome sequence of the *H. influenzae* Rd strain. For all of the 3-6 nucleotide repeats in the *H. influenzae* chromosome, a specific PCR test capable of detecting allelic polymorphism was used. The tri-, penta- or hexanucleotide VNTRs appeared to be relatively stable among the CF *H. influenzae* strains. In general, variability in repeat unit numbers showed concordance with overall genome heterogeneity. However, a problem of this particular approach for studying epidemiology might be that repeat regions are susceptible to "environmental" stress and thus are hyper variable. This may restrict the application of the VNTRs for molecular typing of bacterial strains but it potentially sheds light on virulence potential of a strain.

For instance, most if not all tetranucleotide VNTRs appear to be associated with bacterial virulence genes. Consequently, variation in such repeat unit numbers may affect the virulence gene involved: a gene is either switched on or off. The nature of these genes varies and ranges from the well known LPS biosynthesis genes, and adhesin and glycosyltransferase encoding genes to several iron binding protein genes. Our study demonstrated that during persistent colonization of CF lung tissue serially occurring alterations in VNTR lay-out can be observed in the absence of other changes as assessed by pheno- or genotyping techniques. This observation indicates that the repeat loci may vary independently from major chromosomal polymorphisms. Apparently, different sub-clones of *H. influenzae* develop during colonization. These sub-clones share the overall genome organization but may differ in their virulence gene repertoire. This phenomenon is probably due to the interplay between bacterial replication and human defence mechanisms. The general conclusion of our study is that during persistent colonization of CF tissue, *H. influenzae* displays complicated population dynamics. Strains can be replaced by different genotypes but structural changes within the genome of a single, persisting strain can also lead to the adaptation of virulence factor expression. The micro environment within the CF lung may facilitate selection of this type of subclone and predispose the patient to those strains that are optimally adapted to the CF niche.

A logical conclusion to the above findings is that the CF lung provides a dynamic microbiological environment. Strains come and go, with some persevering for prolonged periods. Although species diversity is observed on occasion, the majority of cultures only yield a single species in abundant quantities. This seems contradictory and for that reason we compared conventional and molecular methods for the detection of bacterial pathogens. (chapter 7) The yield of PCR, cloning and hybridization with species-specific oligonucleotide probes for the detection of *S. aureus*, *H. influenzae* and *P. aeruginosa* was analyzed and compared to the results of routine microbiological culture. This technique demonstrates for the first time the presence of a combination of pathogenic bacterial DNA in the sputum of CF patients, while the conventional methods yielded a single species only. Thus, routine microbiological cultures performed on sputum samples from CF patients do not appear to be sufficient and frequently do not even fully concur with findings of simple tests such as Gram-staining. Shortcomings of the microbiological analysis by cultivation of sputum samples of CF patients have been documented in the literature, especially with regard to *H. influenzae*. In

almost all cases only one bacterial species was documented by culture. In most of the *Pseudomonas* spp. culture-positive sputa, *S. aureus* and *H. influenzae* failed to grow. This observation argues for changes in the diagnostic protocol. Unfortunately, the molecular technique is not able to distinguish between viable and dead organisms, thus the comparison with culture may not be relevant. The presence of a combination of pathogenic bacterial DNA in the sputum of CF patients, together with the idea that it is unlikely that bacterial DNA could persist in the absence of viable bacteria (note the high levels of sputum production in these patients), suggest that we are detecting rDNA from viable and consequently potentially pathogenic bacterial populations. The microorganisms detected by the molecular approach are likely to be alive at the moment of harvesting the clinical material. The colonization patterns of the CF patients were also evaluated. In most of the patients *S. aureus* was cultured at a younger age. *H. influenzae* was cultured only in one patient. Combined with the results from other aspects of our study described in previous chapters, these patterns imply that once a CF patient is colonized with one of the three pathogens, he or she will remain colonized for a long time despite the use of antimicrobial therapy. Even when the pathogen is not detected by conventional techniques, it may still be present. Further studies for the clinical relevance of these findings, and whether or not they should influence therapy and prophylaxis, are needed.

In conclusion, we suggest that current microbiological diagnostics and therapies may be inadequate, and thus molecular procedures should be futher developed and applied in this setting.

Summary en samenvatting

SUMMARY

Cystic Fibrosis is a autosomal recessive hereditary disease, classically recognized by airway infection, exocrine pancreatic insufficiency and elevated sweat chloride. Early respiratory tract pathogens include *Staphylococcus aureus* and *Haemophilus influenzae; Pseudomonas aeruginosa,* particularly mucoid variants, appears in greater prevalence with increasing age. To study the chronic colonization patterns in more detail, many studies typing these pathogens, have been performed. Phenotyping of mucoid *P. aeruginosa* proved to be of limited value. Despite this, many epidemiological studies, using phenotyping, about the colonization of *P. aeruginosa* have been done. In contrast, little is known of colonization patterns of *S. aureus* and *H. influenzae.* With the 'new' molecular techniques we hoped to enhance the insight into the epidemiology of the main pathogens of the respiratory tract of cystic fibrosis patients. Before these techniques could be used, however, we first had to study which technique was best suited for this purpose and to determine its discriminatory power.

Chapter 2 described the characteristics of *P. aeruginosa* isolates pairs recovered from 29 cystic fibrosis patients over an interval of 29 months or more. The fifty-eight isolates were serotyped, pyocin-typed, and subjected to arbitrary primed polymerase chain reaction (AP-PCR) analysis and pulsed field gel electrophoresis (PFGE) analysis. The results indicated that AP-PCR and PFGE showed the greatest strain discriminating ability. AP-PCR showed an advantage in that all isolates were typeable while four of the 58 isolates were not typeable by PFGE, perhaps because of nuclease activity. Comparing the results from both DNA typing techniques generated 15% discordance. Overall, molecular typing techniques showed to be superior to phenotyping for *P. aeruginosa.* In this chapter we further showed that AP-PCR is a fast and useful technique for determining clonality among *P. aeruginosa* isolates. However, the interpretation of data and comparative analysis of PFGE and AP-PCR results necessitated additional (international) standardization and the development of practical guidelines.

To resolve questions about data interpretation, **chapter 3** described a study of eighty-seven strains of *P. aeruginosa.* These strains were typed by random amplification of polymorphic DNA (RAPD) and PFGE. Strains were clustered on the basis of epidemiological data and on

the basis of interpretative criteria for the PFGE analysis, implying that separate types should be differing in more than six fragments. In RAPD assays using the enterobacterial repetitive intergenic consensus sequence ERIC2 primer, and while ignoring single band differences, RAPD-determined grouping of *P. aeruginosa* is congruent with that obtained by PFGE. Consequently, RAPD can be used as a first screen in epidemiological characterization of *P. aeruginosa*.

To study the epidemiology of *P. aeruginosa* among cystic fibrosis siblings, *P. aeruginosa* isolates from sputum samples of 6 pairs of siblings were analyzed longitudinally over a period of 20-126 months.(**Chapter 4**) The isolates were analyzed by DNA fingerprinting using AP-PCR. Two of six pairs of siblings were colonized by identical genotype. A *P. aeruginosa* genotype persisted in a CF patient for over ten years. No overlap in *P. aeruginosa* genotypes between families was encountered. The results of the study demonstrate that *P. aeruginosa* cross-infection or acquisition of the same strain from an identical environmental source exists within the family situation, but does not always result in a long-term colonization by identical genotypes in all CF family members.

In **chapter 5** the epidemiology of *S. aureus* colonized CF patients was studied. *S. aureus* isolates from six unrelated CF patients and six pairs of CF siblings were genetically typed by AP-PCR assays. In most of the patients long-term colonization with a single genotype was observed. One common genotype was found in six patients, two of whom formed a family pair. Identical genotypes were shared by siblings, this suggests intra-familiar transmission or the presence of a common environmental source. The results of the study demonstrate that in most of the CF sibling pairs different genotypes of *S. aureus* caused the ultimate long-term colonization despite cross-infection or identical common environmental source.

To investigate the polymorphism in variable number of tandem repeat (VNTR) loci, serial sputum isolates of *H. influenzae* were obtained from cystic fibrosis patients (**chapter 6**). All strains were genetically characterized by random amplification of polymorphic DNA (RAPD) analysis and strains from two patients were analyzed for polymorphism in the major outer membrane protein (MOMP) profile. For analysis of repeat number variability, a locus could

contain tri- or tetra- or penta- and hexanucleotide repeat units. Most of the regions could be associated with bacterial virulence genes. It appeared that some of the VNTRs were more stable than others. Several of the repeats showed variation, although there were no changes in major outer membrane protein or random amplification of polymorphic DNA types. In conclusion, a persisting *H. influenzae* strain changes its virulence gene regions of the chromosome during colonization of the cystic fibrosis lung, independent of the lack of major chromosomal polymorphism.

It is known that microbiological cultuvation of CF sputa is difficult because *P. aeruginosa* is able to suppress the growth of *S. aureus* and *H. influenzae* can be missed through overgrowth by *P. aeruginosa*. In this study, six sputa of different cystic fibrosis (CF) patients were evaluated using routine microbiological culture and extensive molecular methods (**chapter 7**). Bacterial genes for the small sub-unit ribosomal RNA (ssu rRNA) were specifically amplified from DNA extracted from the sputum samples, cloned and characterized by hybridization and DNA sequencing. Oligonucleotide hybridization was performed with five probes, specific for gram positives and gram negatives in general and the main pathogens for the CF patient (*S. aureus*, *P. aeruginosa* and *H. influenzae*). 2558 clones from six sputa were hybridized correctly. For a single sputum sample the results of microbiological culture and molecular methods were fully congruent. In the other five sputa, discrepancies for *S. aureus* and/or *H. influenzae* were documented. Although *S. aureus* DNA and *H. influenzae* DNA were detected in three and four sputa, respectively, strains were not cultured. Unfortunaly, the PCR approach is not capable of distinguishing viable from dead bacteria. All of the CF patients had a history of *S. aureus* infections, while one of the CF patients once had cultivable *H. influenzae* in the sputum as well. A number of clones for probe-unidentified gram negative or gram positive bacterial species were further analyzed by sequencing and potential pathogens were identified. Routine culture of sputum frequently seems to be a mono-specific exacerbations, our molecular data indicate that the other CF related pathogens appear to be persistently present as well. Overall, the routine culture for bacterial pathogens from CF sputa yields limited microbiological information since it frequently fails to identify a number of pathogenic bacterial species that are potentially present in the lungs of CF patients.

SAMENVATTING

Cystic fibrosis (CF) is een recessieve autosomale aandoening. De klassieke trias voor CF bestaat uit luchtweginfecties, exocriene pancreas insufficiëntie en verhoogde chloride uitscheiding in zweet. Op jonge leeftijd worden de infecties van de tractus respiratorius veroorzaakt door *Staphylococcus aureus* en *Haemophilus influenzae*. De prevalentie van *Pseudomonas aeruginosa* infecties, vooral veroorzaakt door de mucoïde vorm, neemt toe op latere leeftijd. In het verleden is met behulp van fenotypering de epidemiologie van de kolonisatie van *P. aeruginosa* bestudeerd. Het is uit de literatuur bekend dat deze typering van *P. aeruginosa* zijn beperkingen heeft, onder andere veroorzaakt door het mucoïde karakter. Wat betreft de kolonisatie van *S. aureus* en *H. influenzae* is niet veel bekend. De opzet van de studie was om met behulp van de 'nieuwe' moleculaire technieken een beter inzicht te krijgen in de "kolonisatie" epidemiologie van de belangrijkste pathogenen van de tractus respiratorius van de CF patiënt. Echter, voordat de technieken gebruikt konden worden werd eerst bestudeerd welke techniek het meest geschikt was voor gebruik in de studie en diende tevens het onderscheidend vermogen van de technieken vastgesteld te worden.

In **hoofdstuk 2** werd de typering van gepaarde *P. aeruginosa* isolaten, afkomstig van 29 CF patiënten en afgenomen met een interval van 29 maanden of meer beschreven. De 58 isolaten werden geanalyseerd door middel van serotypering, pyocine-typering en de moleculaire technieken, arbitrary primed polymerase chain reaction (AP-PCR) en pulsed field gel electrophoresis (PFGE). De resultaten toonden aan dat AP-PCR en PFGE een groot onderscheidend vermogen hebben. Het voordeel van AP-PCR ten opzichte van PFGE was dat alle isolaten typeerbaar waren in tegenstelling tot PFGE waarbij 4 isolaten mogelijk door nuclease activiteit niet geanalyseerd konden worden. De resultaten van beide DNA technieken laten een discrepantie zien van 15%. Samenvattend, voor *P. aeruginosa* geldt dat moleculaire typeringstechnieken een beter discriminerend vermogen hebben dan fenotyperingstechnieken. Echter, om de interpretatie van de gegevens en de onderlinge vergelijking van de PFGE en AP-PCR resultaten te kunnen standaardiseren diende nog meer onderzoek verricht te worden zodat er richtlijnen voor adequate data interpretatie vervaardigd kunnen worden.

Om richtlijnen te maken voor de interpretatie van AP-PCR patronen werden in **hoofdstuk 3** 87 *P. aeruginosa* isolaten getypeerd met zowel AP-PCR als PFGE. De isolaten waren geclusterd op basis van de epidemiologische gegevens en op basis van het uit de literatuur afkomstige interpretatieve criterium voor PFGE-analyse. Dit criterium was: niet identieke isolaten verschillen in 6 of meer fragmenten. Met de vergelijking tussen de PFGE getypeerde isolaten en de gevonden resultaten met AP-PCR wordt er getracht een criterium op te stellen voor de interpretatie van de AP-PCR data. De AP-PCR analyse van *P. aeruginosa* gebruik makend van de primer enterobacterial repetive intergenic consensus2 (ERIC2) primer bleek identiek aan PFGE als een verschil van 1 enkele fragment genegeerd werd. In verband met het experimenteel gemak en de overeenkomst tussen de AP-PCR en de PFGE data, gebruikmakend van het vastgesteld criterium, bleek AP-PCR een goede screeningsmethode voor epidemiologische onderzoek van *P. aeruginosa*.

De epidemiologie van *P. aeruginosa* afkomstig van gezinsleden lijdend aan CF werd in **hoofdstuk 4** bestudeerd. Voor de typering van de isolaten werd gebruik gemaakt van AP-PCR. De *P. aeruginosa* isolaten, gekweekt uit het sputum van 6 paar gezinsleden, over een periode variërend van 20-126 maanden werden geanalyseerd. Twee van de zes paar gezinsleden bleken gekoloniseerd te zijn met een genotypisch identieke *P. aeruginosa* stam. Tussen de diverse families is geen overdracht van *P. aeruginosa* genotypen gevonden. In 1 CF patiënt persisteerde een *P. aeruginosa* genotype gedurende ruim 10 jaar. De resultaten van deze studie geven aan dat in een gezinssituatie kruisinfectie en/of acquisitie van hetzelfde isolaat, mogelijk vanuit een gemeenschappelijke omgevingsbron, bestaat, maar dat dit niet altijd leidt tot een langdurige kolonisatie met identieke genotypen binnen deze gezinnen.

In **hoofdstuk 5** is de epidemiologie van *S. aureus* in CF patiënten bestudeerd. Met behulp van AP-PCR werden *S. aureus* isolaten afkomstig van 6 niet gerelateerde CF patiënten en 6 paar gezinsleden met CF getypeerd. In de meeste patiënten werd langdurige persistentie van een bepaald genotype waargenomen. Eén genotype werd bij zes patiënten gevonden, waarvan er slechts 2 een familiepaar vormden. Uitwisseling van identieke genotypen vond plaats bij familieleden gedurende een bepaalde tijd. Dit pleit voor een intra familiaire transmissie of aanwezigheid van een gemeenschappelijke omgevingsbron. De studie resultaten impliceren

dat de meeste gezinsleden uiteindelijk langdurig gekoloniseerd zijn met "hun eigen" *S. aureus* genotype ondanks potentiële kruisinfectie of een gemeenschappelijke omgevingsbron.

Ook de epidemiologie van *H. influenzae* bij CF patiënten is relatief spaarzaam onderzocht. In **Hoofdstuk 6** werd genetische karakterisering van seriële *H. influenzae* isolaten uit het sputum van 8 CF patiënten beschreven. De isolatie periode varieerde van 1 tot 8 jaar. De *H. influenzae* isolaten werden getypeerd middels AP-PCR. Bij 2 patiënten werd tevens gekeken naar de variatie in het buiten membraaneiwit (major outer membrane protein, MOMP) patroon. De typeringen (AP-PCR, MOMP) toonden aan dat persistentie van isolaten bij enkele patiënten voorkwam. Van *H. influenzae* is bekend dat de regulatie van virulentie gen expressie voor een deel kan worden toegeschreven aan variatie in de lengte van gebieden van repetitief DNA. De lengte veranderingen geven aanleiding tot verstoring van leesramen van genen of leiden tot aantasting van de structuele integriteit van promotoren. De meeste loci van repetitief DNA zijn geassocieerd met eenduidige bacteriële virulentie factoren zoals LPS biosynthese enzymen, adhesines, glycosyltransferases, ijzer bindingsproteinen, methyltransferase en enzymen betrokken bij synthese van fimbriae. De resultaten van deze studie laten zien dat er variatie in variable number of tandem repeats (VNTR's) kan bestaan zonder dat er veranderingen optreden in het buiten membraaneiwit patroon of het AP-PCR patroon. Hieruit concludeerden wij dat een stam in staat is gedurende de kolonisatie van de CF long om via toevallige variatie in de VNTRs het virulentie potentieel te moduleren en mogelijkerwijs zelfs aan te passen aan veranderende gastheer condities.

Het microbiologisch analyseren van CF sputum is niet eenvoudig. *P. aeruginosa* is in staat de groei van *S. aureus* te onderdrukken en *H. influenzae* kan gemist worden door overgroei van *P. aeruginosa*. In **hoofdstuk 7** werden zes sputa van verschillende CF patiënten zowel met behulp van routinematige microbiologische kweek technieken als moleculaire technieken bestudeerd. Genen voor het bacterieel 16S RNA (small sub-unit ribosomal RNA ofwel ssu rDNA) werden geamplificeerd met behulp van breed-spectrum primers en de PCR producten werden gekloneerd. De herkomst van individuele, bacteriesoort specifieke kloons werd vervolgens vastgesteld met behulp van hybridisatie en DNA sequencing. Hybridisatie is verricht met twee algemene probes coderend voor gram-positieve en gram-negatieve bacteriën.

Daarnaast is gewerkt met drie additionele probes die specifiek zijn voor *S. aureus*, *P. aeruginosa* en *H. influenzae*. Van één sputum waren de resultaten van de microbiologische kweek en de moleculaire methode volledig overeenkomstig. In de overige vijf sputa werden discrepanties betreffende de aanwezigheid van *S. aureus* en/of *H. influenzae* waargenomen. Ondanks het feit dat DNA van *S. aureus* en *H. influenzae* in drie respectievelijk vier sputa werd aangetoond, konden deze isolaten niet gekweekt worden. Al deze CF patiënten hadden in hun voorgeschiedenis een *S. aureus* infectie doorgemaakt maar slechts bij één CF patiënt werd ooit *H. influenzae* uit het sputum gekweekt. Een aantal klonen die met de gram-negatieve probe of met de gram-positieve probe aankleurde maar niet geïdentificeerd werden door één van de drie specifieke probes werden verder geanalyseerd door middel van sequencing. Hierbij werden een aantal potentiële pathogenen, die niet eerder gekweekt werden zoals *Escherichia coli*, *Klebsiella pneumoniae* en *Pseudomonas citronellosis*, geïdentificeerd. Tijdens het kweken van CF sputa worden doorgaans mono-culturen van één van de drie 'klassieke' pathogenen gevonden. Echter, de moleculaire data geven aan dat ook andere CF gerelateerde pathogenen op dat moment nog aanwezig kunnen zijn. Wij concluderen dat de routine kweektechnieken voor de CF pathogenen zijn beperkingen hebben omdat zij frequent falen in het isoleren van pathogene bacteriën die met de moleculaire technieken wel aangetoond kunnen worden en die mogelijk klinisch relevant zijn.

Nawoord en curriculum vitae

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Perc, ik weet hoe je over een nawoord denkt, dus zal ik het kort houden. Bedankt.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 10 mei 1960 te Berg en Dal. Na het behalen van het middelbare school diploma aan het Canisius-Mater Dei college te Nijmegen werd in 1981 begonnen aan de opleiding Hogere Laboratorium Onderwijs aan het dr. ir Ghijsen instituut te Utrecht. In 1986 werd het diploma behaald. In datzelfde jaar werd aangevangen met een part-time baan als medisch microbiologisch analiste in ziekenhuis Berg en Bosch te Bilthoven en tevens werd er gestart met de studie Geneeskunde aan de Rijksuniversiteit te Utrecht. In december 1987 nam ze ontslag waarna in juni 1990 het doctoraal examen geneeskunde werd behaald. Aansluitend na het artsexamen werd er begonnen met de opleiding tot medisch microbioloog aan het Dijkzigt ziekenhuis te Rotterdam (opleider Prof. dr. HA Verbrugh). Het onderzoek dat in dit proefschrift wordt beschreven, werd begonnen tijdens de opleiding. Inschrijving in het specialistenregister volgde op 1 april 1997. Sinds 1997 is zij werkzaam als arts-microbioloog in het Bosch Medicentrum en het Carolus ziekenhuis te 's-Hertogenbosch.