# Longitudinal Assessment of *Pseudomonas aeruginosa* in Young Children with Cystic Fibrosis

**Jane L. Burns,[1] Ronald L. Gibson,[2,3] Sharon McNamara,[3] Darlene Yim,[1] Julia Emerson,[3] Margaret Rosenfeld,[2,3] Peter Hiatt,[4] Karen McCoy,[5] Robert Castile,[5] Arnold L. Smith,[6] and Bonnie W. Ramsey[2,3]**

*Divisions of [1]Infectious Disease and [2]Pulmonary Medicine and [3]Cystic Fibrosis Center, Department of Pediatrics, University of Washington and Children's Hospital and Regional Medical Center, Seattle; [4]Department of Pediatrics, Baylor College of Medicine, Houston, Texas; [5]Department of Pediatrics, Columbus Children's Hospital, Columbus, Ohio; [6]Department of Molecular Microbiology and Immunology, University of Missouri–Columbia, Columbia*

***Pseudomonas aeruginosa*** **lung infection is an important cause of morbidity and mortality in cystic fibrosis (CF). Longitudinal assessment of the phenotypic changes in *P. aeruginosa* isolated from young children with CF is lacking. This study investigated genotypic and phenotypic changes in *P. aeruginosa* from oropharynx (OP) and bronchoalveolar lavage fluid (BALF) in a cohort of 40 CF patients during the first 3 years of life; antibody response was also examined. A high degree of genotypic variability was identified, and each patient had unique genotypes. Early isolates had a phenotype distinct from those of usual CF isolates: generally nonmucoid and antibiotic susceptible. Genotype and phenotype correlated between OP and BALF isolates. As determined by culture, 72.5% of patients demonstrated *P. aeruginosa* during their first 3 years. On the basis of combined culture and serologic results, 97.5% of patients had evidence of infection by age 3 years, which suggests that *P. aeruginosa* infection occurs early in CF and may be intermittent or undetectable by culture.**

Pulmonary infection is the most common cause of morbidity and mortality in cystic fibrosis (CF) [1]. The bacteriology of CF lung infections is well defined in older children and adults, because expectorated sputum is available for culture [2]. Unfortunately, children <6 years old cannot reliably produce sputum [1], and oropharyngeal (OP) cultures may not predict lower airway colonization [3–5]. Thus, the microbiology of pulmonary infections in this younger age group is based on cultures of specimens obtained during autopsy, during a period of intubation for severe disease or for an elective surgical procedure, or during bronchoscopy for clinical indications. The timing of such specimens is unlikely to reveal information about the acquisition of specific pathogens and often cannot be correlated with the immune response or clinical course.

Several recent studies have used bronchoalveolar lavage (BAL) to obtain specimens for culture from the lower airway in young children with CF [3, 4, 6–11]. Most of these studies were cross-sectional and did not monitor the timing of acquisition of specific pathogens or the evolution of genotypic or phenotypic changes in *Pseudomonas aeruginosa* [12]. To address these concerns, we performed a multicenter study of CF lung infection in the first 3 years of life. Specimens for serial upper and lower airway cultures and serology were obtained. Our primary aims were to identify the age at which young children with CF first acquire *P. aeruginosa,* to determine whether unique bacterial strains are found early in colonization, to examine concordance between isolates from upper and lower airway cultures, and to characterize surrogate markers of lower airway infection in this age group.

## Materials and Methods

*Study design.* The design was a prospective, longitudinal cohort study involving scheduled observation and data collection among young infants with CF who were enrolled at 3 CF centers: Children's Hospital and Regional Medical Center in Seattle, Children's Hospital in Columbus, Ohio, and Texas Children's Hospital in Houston. Study inclusion criteria were (1) age ⩽15 months at enrollment; (2) diagnosis of CF, based on 2 of the following: (a) sweat chloride, by quantitative pilocarpine electrophoresis, ⩾60 mEq/L, (b) 2 clinical features consistent with CF, or (c) genetic testing demonstrating 2 mutations associated with CF; and (3) informed consent provided by parents or legal guardian. Subjects were excluded if they had a serious coexisting condition that would preclude annual bronchoscopy with sedation.

Bronchoscopy with BAL was performed annually. Because enrollment could occur up to age 15 months, the initial bronchoscopy could be between 11 and 15 months of age. Subsequent bronchos-

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This study was approved by the institutional review boards of all the participating centers, and informed consent was received from the parents of the participating study subjects.

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Reprints or correspondence: Dr. Jane L. Burns, Division of Infectious Disease, CH-32, Children's Hospital and Regional Medical Center, 4800 Sand Point Way NE, Seattle, WA 98105 (jburns@chmc.org).

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copies were performed annually, at age 24 and 36 months (±1 month). Clinical and laboratory evaluations were performed at enrollment and at 3-month intervals, until the third bronchoscopy. Laboratory studies included OP culture and anti–*P. aeruginosa* serology. Data collection was completed at the time of the bronchoscopy at age 3 years. Antibiotic prescribing practices were not standardized as part of the study design but were recorded.

*Bronchoscopy and BAL fluid (BALF) culture.* The choice of flexible or rigid bronchoscopy was made on the basis of available bronchoscopists at each institution, rather than on the basis of subject characteristics. With few exceptions, flexible bronchoscopy was used in Columbus ($n = 36$ bronchoscopies performed) and in Houston ($n = 36$ bronchoscopies), whereas rigid bronchoscopy was used in Seattle ($n = 37$ bronchoscopies). Flexible fiberoptic bronchoscopy for lavage was performed via the transnasal route, using a 3.6-mm pediatric scope, under intravenous sedation with midazolam and morphine. Topical anesthesia of the larynx, trachea, and carina was achieved, as needed, with 1% lidocaine, with a maximum dose of 4 mg/kg. The suction channel was not used until the tip of the bronchoscope was below the carina. The bronchoscope was wedged in the lingula and was lavaged with 3 1 mL/kg aliquots of nonbacteriostatic saline (maximum, 10 mL). Returned BALF was immediately placed on ice. Rigid bronchoscopy was performed under general anesthesia, with topical 1% lidocaine. A 10-French catheter, with the Murphy eye removed, was passed through the bronchoscope and was wedged for BAL. Suction was avoided until the catheter was wedged and the saline had been instilled. Lavage and BALF processing were performed as described above.

*Oropharyngeal culture.* A cotton-tipped swab was used to collect the specimen from the posterior oropharyngeal wall and tonsillar pillars. The OP samples were obtained within 1 h before bronchoscopy at annual visits and before chest physiotherapy and postural drainage.

*Microbiology.* All cultures were processed in a centralized microbiology laboratory at Children's Hospital and Regional Medical Center in Seattle. Samples were shipped on wet ice packs via overnight mail. OP samples were inoculated directly, but BALF was processed quantitatively, using a modification of the technique of Wong et al. [13]. *P. aeruginosa* were isolated on MacConkey and cetrimide agar plates, and identification was confirmed by pigment production and growth on cetrimide agar or by standard biochemical testing [2]. All bacterial isolates were frozen at −80° C after their removal from the primary culture plate.

*Susceptibility testing.* Broth microdilution MICs of all *P. aeruginosa* isolates were performed immediately after bacterial isolation, using the semiautomated Sensititre system (AccuMed). The antibiotics tested were amikacin, gentamicin, tobramycin, aztreonam, ceftazidime, ticarcillin, and ciprofloxacin.

*Phenotypic characterization.* All *P. aeruginosa* isolates were examined for mucoidy, colony morphology (regular or irregular), and pigment production. All isolates were evaluated by the same experienced individual (D.Y.) after overnight growth on cetrimide agar. Pigment production was scored according to a numeric scale, based on paint chips. Although pigment production was scored numerically, the pigments were also classified into broad categories of greens, tans, and pinks. Whenever possible, isolates were scored before being passed or frozen, and, if frozen, they were scored on the first passage out of the frozen stock. A change in any one of the above characteristics was considered a difference in phenotype.

*Genotyping.* Genotyping of all *P. aeruginosa* isolates was performed by using restriction fragment length polymorphisms (RFLPs), probing with the exotoxin A gene [14]. Each phenotypically distinct isolate was subcultured and was grown in broth culture. Total genomic DNA was extracted [15] and was digested separately with *Bam*HI and *Bgl*II, electrophoresed through 0.8% agarose, and transferred to a nylon membrane [16]. The membrane was probed with the digoxigenin-labeled (Boehringer-Mannheim) *Eco*RV-*Bgl*II fragment (∼1 kb) of plasmid pRGI [14], and bound probe was detected by chemiluminescence. All isolates from each individual patient were run on the same gel, enabling comparison of banding patterns. Isolates with identical RFLP patterns using both enzymes were considered to be of the same genotype.

*Exotoxin A serology.* Exotoxin A ELISA was performed by using a modification of the technique of Ramsey et al. [4], with 2 μg of exotoxin A coated on the well. Serum samples from 16 healthy control children <6 years of age were used to confirm the appropriateness of the cutoff value for seropositivity used in the previous study.

*Immunoblot analysis.* Antibodies directed against whole-cell proteins from *P. aeruginosa* were detected by immunoblot. Each patient's own organisms served as the source of antigens. If a patient had >1 genotype isolated, each was used individually. If a patient had no *P. aeruginosa* isolated, laboratory strain PAO1 was used. Total bacterial proteins were prepared by a modification of the technique of Hancock and Nikaido [17]. Similar results were obtained on immunoblot analysis whether whole-cell proteins or outer membrane preparations were used (data not shown). Protein concentration was quantitated by using the Bradford protein assay, and 25 μg per well was loaded. Proteins were electrophoresed on a 12% SDS polyacrylamide gel and were transferred to nitrocellulose membranes overnight. Dried membranes were cut into 5-mm strips, and the strips were blocked with 5% dried nonfat milk in PBS (pH 7.0), incubated with patient serum (1:5000 dilution), washed, and incubated with goat anti–human IgG horseradish peroxidase conjugate (1:10,000 dilution). Blots were developed by using SuperSignal HRP substrate (Pierce) and were visualized on x-ray film. Reactive membrane proteins were compared with known *P. aeruginosa* antigens [18]. Serum samples lacking exotoxin A antibodies, from 8 healthy children between 1 month and 6 years of age, served as negative controls. An individual patient's blots were all examined together in temporal order, and patterns were identified. Each time point was viewed as a part of a continuum, rather than as an individual point. If a patient had a single band or very few bands noted at an early time point that later became more distinct or part of a larger banding pattern, those bands were identified as representing immunogenic proteins. If no distinct bands were identified, or if faint or isolated bands did not fit with later patterns, the serum at that time point was identified as lacking antibodies to *P. aeruginosa.*

*Statistical methods.* Data analysis included using descriptive measures (i.e., numbers and percentages for categorical data and means, SDs, medians, and ranges for continuous variables) to summarize study variables . Comparisons of susceptibility for *P. aeruginosa* isolates from BALF and OP cultures were performed by categorizing MIC results for a given antibiotic as susceptible or

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resistant and determining whether results were concordant for each pair of cultures. The diagnostic accuracy of OP cultures relative to BALF cultures was evaluated by calculations of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), with corresponding 95% binomial confidence intervals (CIs). Each culture was categorized with respect to the presence or absence of *P. aeruginosa,* and results for OP versus BALF cultures were cross-classified in 2 × 2 tables.

## Results

*Patient population.* Between March 1993 and April 1996, a total of 42 subjects were enrolled, 14 at each of the 3 centers. Two subjects were withdrawn before the first bronchoscopy (at 12 months) and were excluded from further analysis. Two additional subjects had been withdrawn by 24 months, and 2 more were lost to follow-up (one after the 24-month visit, one after the 33-month visit). Mean age at diagnosis of CF was 3.9 months (range, 1 week to 8.5 months), and mean age at enrollment was 11.4 months (range, 2.5–15.5 months). Twenty-four subjects were male; none were siblings. All but one patient was genotyped. Twenty-four patients were homozygous and 13 heterozygous for Δ*F508;* the remaining 4 patients had other mutations.

P. aeruginosa *culture results.* One hundred nine bronchoscopies were completed in 40 patients; one sample was inadvertently not cultured. Of the 108 BALF cultures performed, 40 were performed at 1 year of age, 35 at 2 years, and 33 at 3 years. Lower airway quantitative cultures were analyzed for any density of *P. aeruginosa.* Seven (18%), 12 (34%), and 11 (33%) subjects had *P. aeruginosa* isolated from BALF at 1, 2, and 3 years of age, respectively. At least 1 *P. aeruginosa* isolate was detected in 29 (72.5%) of the 40 patients whose specimens were cultured: 11 (27.5%) from OP only and 18 (45%) from both OP and BALF at some time during the study. Altogether, 193 *P. aeruginosa* isolates from these patients were identified: 45 from BALF and 148 from OP.

P. aeruginosa *genotype analysis.* The genotypes of all *P. aeruginosa* isolates from OP and BALF cultures were compared within and between patients. Each individual had a single or multiple unique genotypes, which suggests that there is not a single specific clone that is the initial isolate in infants with CF.

Of the 11 patients with *P. aeruginosa* from OP only, 6 had a single genotype, 4 had 2 genotypes, and 1 had 3 genotypes isolated during the study period. In the 5 patients with multiple genotypes from OP culture, time from initial isolation to the second genotype averaged 5.4 months (range, 0–18 months). In the 6 patients with a single genotype, an average of 13.5 months (range, 3–24 months) elapsed between initial isolation of *P. aeruginosa* and the conclusion of the study.

Eighteen patients had *P. aeruginosa* isolated from both OP and BALF cultures. Five of these patients had a single genotype identified during the study period. The remaining 13 patients had multiple genotypes, including 10 patients with 2 genotypes, 2 with 3, and 1 with 4 genotypes; the results from those 13 patients are listed in table 1. In the patient with 4 genotypes (patient 202), 3 genotypes were found only in the OP culture, and a single different genotype was recovered only from BALF culture. Figure 1 illustrates a representative patient (patient 211) with 3 genotypes. This patient had a single genotype, in both OP and BALF samples, from 1 year of age. A second, unique genotype was isolated from an OP culture at 21 months of age, followed by a third, in both OP and BALF cultures, at 2 years of age. This latter genotype persisted in subsequent cultures of OP and BALF throughout year 3.

There was concordance between the genotypes of the first isolates from OP and BALF cultures (some of which were not isolated simultaneously) in 7 of the 13 patients and discordance in the other 6. Including the 5 patients with a single genotype, there was genotypic concordance between initial upper and lower airway isolates in 12 of the 18 patients with BALF cultures yielding *P. aeruginosa.*

In the 13 patients with multiple genotypes from OP and BALF cultures, an average of 9.9 months (range, 3–21 months) elapsed between the identification of the first *P. aeruginosa* genotype and the second. In the 5 patients with a single genotype from upper and lower airway cultures, a mean of 13.8 months (range, 0–27 months) elapsed from the time of the initial isolation of *P. aeruginosa* to the end of the study.

There were 18 annual visits (in 14 patients) at which concurrent OP and BALF cultures both yielded *P. aeruginosa.* The genotypes of the OP and BALF isolates were identical in all but one: patient 102, at 36 months, had 2 genotypes present in the OP culture, and only one of the 2 was recovered from the BALF culture.

P. aeruginosa *phenotype.* The phenotype of each *P. aeruginosa* isolate from each patient was characterized. Overall, 5 of the 29 patients from whom *P. aeruginosa* was ever isolated had a mucoid strain at least once from the upper or lower airway during the first 3 years of life: none in the first year, 3 in the second, and 4 in the third. There were 2 patients in whom a mucoid *P. aeruginosa* was isolated from BALF culture. In patient 6, a mucoid *P. aeruginosa* was isolated from the BALF at 24 months, 3 months after the first OP culture yielded *P. aeruginosa.* In patient 9, the first *P. aeruginosa* isolate (OP) was recovered at 12 months, whereas the first mucoid isolate (OP) was recovered at 21 months and the first mucoid BALF isolate at 24 months (figure 2). Of the other 3 patients with mucoid *P. aeruginosa* isolated from OP cultures only, 2 had mucoid *P. aeruginosa* at their first isolation and one had *P. aeruginosa* first isolated at 15 months, with the first mucoid phenotype at 30 months. Four of the 5 patients with mucoid *P. aeruginosa* had received parenteral or aerosol antibiotics at some time before isolation of the mucoid organism, but there was no clear-cut temporal association with such therapy. Overall, 95% of the patients received parenteral or aerosol antimicrobial therapy on ⩾1 occasions during the study. Both OP and BALF isolates mostly produced green pigmentation

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**Table 1.** Pattern of genotypic variation in 13 patients with >1 genotype of *Pseudomonas aeruginosa* from oropharynx (OP) and bronchoalveolar lavage fluid (BALF) cultures.

| Patient, culture | Visit, months of age | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 33 | 36 |
| Patient 3 | | | | | | | | | | | | |
| OP | — | — | — | — | — | — | G3B | — | — | — | G3C | — |
| BALF | — | — | — | G3A | — | — | — | G3B | — | — | — | G3C |
| Patient 6 | | | | | | | | | | | | |
| OP | — | — | — | — | — | — | G6A | — | — | — | G6C | G6C |
| BALF | — | — | — | — | — | — | — | G6B | — | — | — | — |
| Patient 7 | | | | | | | | | | | | |
| OP | — | — | — | G7A | — | G7B | — | G7B | G7B | G7B | G7B | — |
| BALF | — | — | — | G7A | — | — | — | G7B | — | — | — | — |
| Patient 9 | | | | | | | | | | | | |
| OP | — | G9A | G9A | — | G9A | G9A | G9A, G9B | G9A | G9A | G9A | G9A | G9A |
| BALF | — | — | — | G9A | — | — | — | G9A | — | — | — | G9A |
| Patient 10 | | | | | | | | | | | | |
| OP | — | — | — | G10A | G10A | G10A | G10A | G10A | — | G10A | G10A, G10B | — |
| BALF | — | — | — | — | — | — | — | G10A | — | — | — | G10B |
| Patient 102 | | | | | | | | | | | | |
| OP | — | — | — | — | — | — | — | — | G102A | G102A, G102B | G102A, G102B | G102A, G102B |
| BALF | — | — | — | — | — | — | — | — | — | — | — | G102A |
| Patient 109 | | | | | | | | | | | | |
| OP | — | — | — | — | — | — | — | — | — | G109A | — | G109B |
| BALF | — | — | — | — | — | — | — | — | — | — | — | G109B |
| Patient 111 | | | | | | | | | | | | |
| OP | — | — | — | — | — | — | — | G111B | — | — | — | — |
| BALF | — | — | — | G111A | — | — | — | — | — | — | — | — |
| Patient 202 | | | | | | | | | | | | |
| OP | — | — | — | — | — | — | — | — | G202B, G202C | G202B, G202C | — | G202D |
| BALF | — | — | — | — | — | — | — | G202A | — | — | — | — |
| Patient 203 | | | | | | | | | | | | |
| OP | — | — | — | — | — | G202A | — | G202B | G202B | — | G202B | — |
| BALF | — | — | — | — | — | — | — | G202B | — | — | — | G202B |
| Patient 205 | | | | | | | | | | | | |
| OP | — | — | — | — | G205A | G205A | G205A | G205A | G205A | G205A, G205B | G205A | G205A |
| BALF | — | — | — | — | — | — | — | G205A | — | — | — | — |
| Patient 206 | | | | | | | | | | | | |
| OP | — | — | — | — | — | G206A | — | G206A | — | G206A | G206A, G206B | — |
| BALF | — | — | — | G206A | — | — | — | G206A | — | — | — | — |
| Patient 211 | | | | | | | | | | | | |
| OP | — | — | — | G211A | — | — | G211B | G211C | — | G211C | G211C | — |
| BALF | — | — | — | G211A | — | — | — | G211C | — | — | — | G211C |

NOTE. In genotype designations, "G" (genotype) and patient number are followed by "A" (first unique genotype), "B" (second unique genotype), or "C" (third unique genotype).

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(98% and 99%, respectively), suggestive of pyoverdin and pyocyanin production.

*Upper and lower airway isolate concordance.* Eleven of the 29 patients who had *P. aeruginosa* isolated from OP or BALF culture had a single phenotype identified. Ten patients had 2 phenotypes identified, and 8 patients had ⩾3 phenotypes (maximum, 6 in a single patient). Of the 18 annual visits (for 14 patients) during which *P. aeruginosa* was isolated simultaneously from both BALF and OP cultures, there were 8 visits wherein multiple phenotypes were isolated. In this group, there were 5 instances of simultaneous isolation of dissimilar phenotypes from OP and BALF cultures; however, ⩾1 phenotype was always shared between upper and lower airway isolates.

*Analysis of changes in* Pseudomonas *genotype and phenotype.* Although 18 of the 29 patients from whom *P. aeruginosa* was isolated had changes in genotype or in phenotype, only 14 patients had changes in both genotype and phenotype, and never simultaneously. Patient 9 (figure 2) illustrates the lack of correlation between changes in genotype and phenotype: 21 of the 22 isolates from this patient were a single genotype, including 5 mucoid and 16 nonmucoid isolates. The single isolate of a second genotype was a mucoid OP isolate from year 2 that did not persist in either the upper or lower airway. Thus, genotypic and phenotypic changes appeared to be unrelated events within the same patient.

The timing of genotypic and phenotypic changes was compared with the timing of parenteral or aerosol antibiotic administration and with development of *P. aeruginosa* antibodies. There was no evidence of an association between these factors and the timing of changes in phenotype or genotype.

*Antimicrobial susceptibility.* Antimicrobial susceptibility testing of all 45 BALF isolates and 145 of the 148 OP isolates

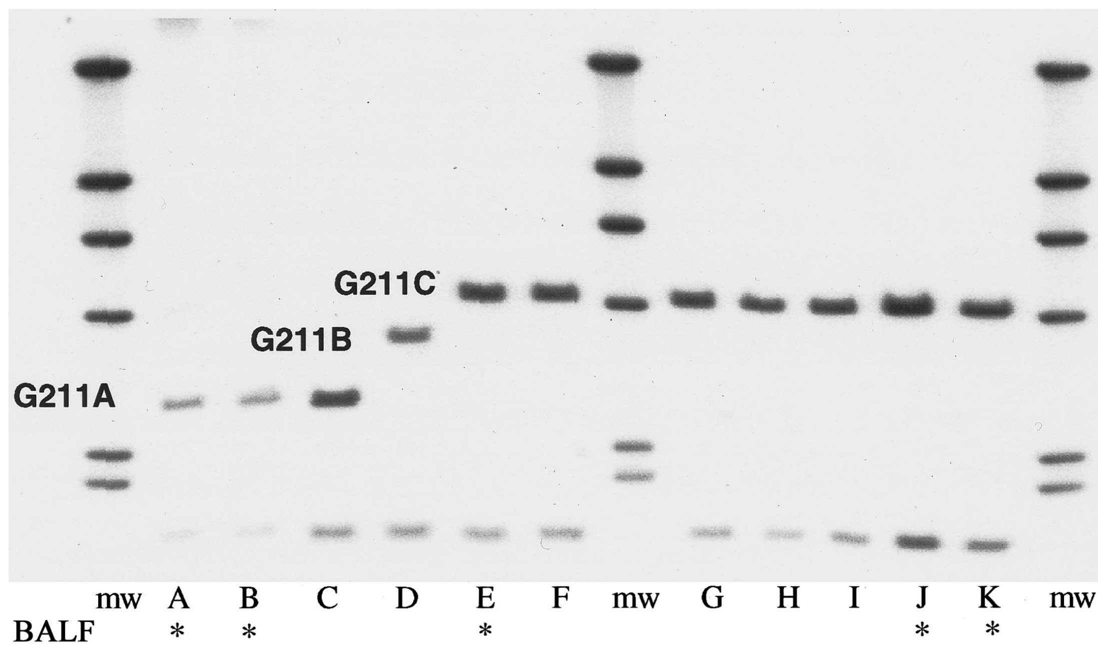


**Figure 1.** Genotyping of 11 upper and lower airway *Pseudomonas aeruginosa* isolates from patient 211, using an exotoxin A gene to probe *Bam*HI digests of genomic DNA. *Lanes A–C,* Year 1; *lanes D–F,* year 2; *lanes G–K,* year 3. *Bronchoalveolar lavage fluid (BALF) isolates *(lanes A, B, E, J,* and *K).* mw, Molecular-weight marker.

of *P. aeruginosa* was performed, to determine whether the susceptibility of OP isolates was predictive of susceptibility patterns in lower airway isolates and whether there was an association between antipseudomonal therapy and development of resistance. Susceptibility to 7 antibiotics was compared for the *P. aeruginosa* isolates obtained at the 18 annual visits during which the organism was isolated simultaneously from both BALF and OP cultures. Of the resulting 126 paired observations, only 4 were discordant with respect to susceptibility to a specific antibiotic. Of these, 3 demonstrated susceptibility in a BALF isolate when the OP isolate was resistant, whereas only one demonstrated resistance in the BALF isolate when the OP isolate was susceptible.

Most *P. aeruginosa* isolates (91% of BALF isolates and 82% of OP isolates) were susceptible to all agents tested. A single patient had 2 isolates of *P. aeruginosa* (one from OP and one from BALF culture at 3 years) that were resistant to all agents tested. This patient had received 5 courses of parenteral antipseudomonal therapy, compared with a mean of 4.7 courses (range, 0–16) in the cohort as a whole. Four BALF isolates from 3 patients were resistant to $\geqslant$1 of the antimicrobial agents tested.

*OP culture results as a potential surrogate marker of lower airway* P. aeruginosa. OP cultures were performed concurrently with BALF cultures and at 3-month intervals in between, so it was possible to correlate cultures from concurrent and preceding quarterly visits. OP cultures performed concurrently with BALF cultures had a better NPV than PPV. Subjects with an OP culture lacking *P. aeruginosa* were unlikely to have that organism detected in BALF culture (NPV, 85%; 95% CI, 76%–92%). Isolation of *P. aeruginosa* from the OP culture was less accurate in predicting lower airway *P. aeruginosa* isolation (PPV, 69%; 95% CI, 48%–86%). Combining the results of 2 OP cultures (concurrent with and 3 months before the BAL culture) yielded the highest predictive values of the measures we analyzed. The NPV of this combination of OP cultures was 97% (95% CI, 86%–100%), and the PPV was 83% (95% CI, 52%–98%).

*Immune response to* P. aeruginosa. The ELISA for exotoxin A antibodies and the immunoblot for antibodies directed against whole-cell proteins from each patient's own organisms were compared with OP and BALF cultures (table 2). The mean and median ages at which antibodies were first detected were younger for both serologic assays than for either culture. Antibodies directed against whole-cell proteins were present in the largest percentage of patients and at the youngest age of the 4 assays. One limitation of this comparison is that BALF cultures were performed only annually, whereas serologic analysis and OP cultures were performed quarterly.

Exotoxin A antibodies were identified in 12 patients (30%) at or before the first annual visit, with 31 patients (78%) eventually found to have exotoxin A antibodies. Antibodies directed against whole-cell proteins were present in 20 patients (50%) at the first study visit, with 36 (90%) eventually found to have such antibodies. The culture and serology results of a representative patient are shown in figure 3.

The patients were divided into 3 groups based on culture status at the end of the study, and serology results were ex-

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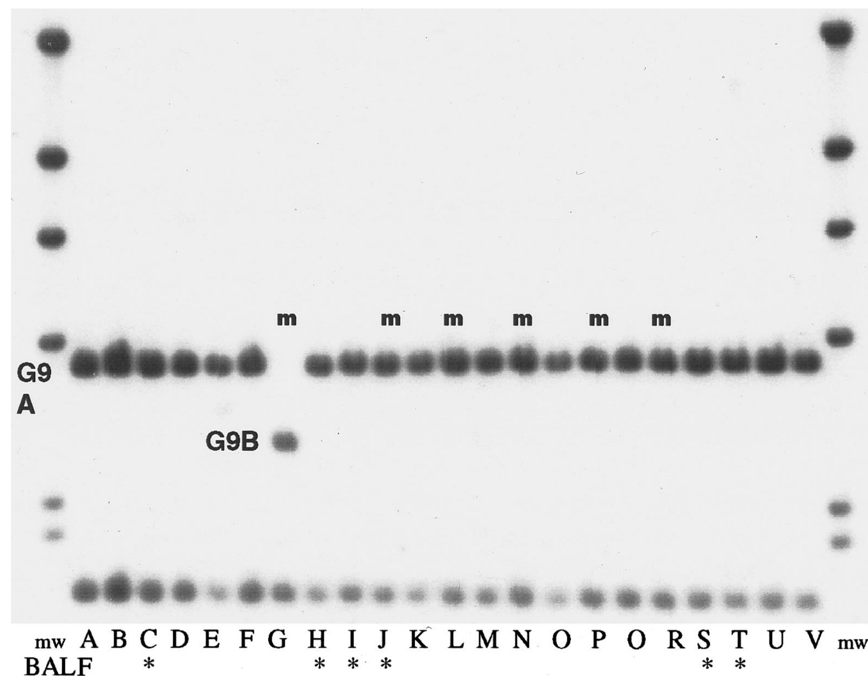


**Figure 2.** Genotyping of 22 upper and lower airway *Pseudomonas aeruginosa* isolates from patient 009, using an exotoxin A gene to probe *Bam*HI digests of genomic DNA. *Lanes A–C,* Year 1; *lanes D–L,* year 2; *lanes M–V,* year 3. *Bronchoalveolar lavage fluid (BALF) isolates *(lanes C, H, I, J, S,* and *T).* An "m" indicates phenotypically mucoid isolates *(lanes G, J, L, N, P,* and *R).* mw, Molecular-weight marker.

amined for each group. *P. aeruginosa* was isolated from BALF during the study in 18 patients, all of whom demonstrated antipseudomonal antibodies; a single patient in this group lacked antibody to exotoxin A, and a different patient lacked antibodies directed against whole cells. There were 11 patients for whom *P. aeruginosa* was cultured only from OP; 10 of these patients had antibodies against exotoxin A, and 9 had antibodies against whole-cell proteins. Only 1 patient did not have detectable antipseudomonal antibodies. The single antibody-negative patient had organisms with 3 different genotypes isolated from OP. There were also 11 patients who did not have *P. aeruginosa* cultured from either site at any time point. Of those, 4 had antibody against exotoxin A, 10 had antibodies against whole-cell proteins, and, overall, 10 patients had evidence of antipseudomonal antibodies. By 3 years of age, only one of the 40 patients lacked evidence of *P. aeruginosa* infection, on the basis of a combination of culture and serologic results.

## Discussion

This longitudinal study examined the evolution of upper and lower airway infection in a cohort of young children with CF. In this population, similar to those of Khan et al. [10], Armstrong et al. [3, 6, 7], and Noah et al. [8], both infection and inflammation occurred early (authors' unpublished data). However, significant controversy remains about whether inflammation is increased at baseline in CF or increased inflammation is the result of early lung infection with *P. aeruginosa* or other CF pathogens. The assessment of both culture and serology status and the longitudinal monitoring of this cohort of young children enable the current study to shed light on this question.

Whether increased inflammation or infection is the initial event in CF lung disease can be answered only if sensitive assays are used to assess infection status. If lower airway culture yielding *P. aeruginosa* is considered to be the reference standard for infection, only 45% of the patients studied here would be considered to be infected. This percentage of infected infants is in the range of those reported in previous studies: ~0%–54% of young children infected with *P. aeruginosa,* depending on age at sampling [6–8, 10, 11]. This large range may be accounted

**Table 2.** Time of first positive *Pseudomonas aeruginosa* culture or serology results.

| Event | No. (%) of patients ever positive[a] | Age (mo) at 1st positive test result in patients ever positive | | |
|---|---|---|---|---|
| | | Mean (SD) | Median | Range |
| BALF culture[b] | 18 (45) | 22.8 (8.9) | 23.7 | 12.1–37.2 |
| OP culture[c] | 29 (73) | 20.7 (8.6) | 18.9 | 5.9–37.2 |
| Exotoxin A antibodies[c] | 31 (78) | 18.4 (7.7) | 15.5 | 9.1–36.5 |
| Whole-cell protein antibodies[c] | 36 (90) | 15.4 (5.8) | 13.7 | 8.7–32.9 |

NOTE. BALF, bronchoalveolar lavage fluid; OP, oropharynx.
[a] Percentages of the 40 patients who completed at least the first annual visit.
[b] Samples for BALF culture were obtained only at annual visits.
[c] Samples for OP culture and serum for antibody analysis were obtained at quarterly visits.

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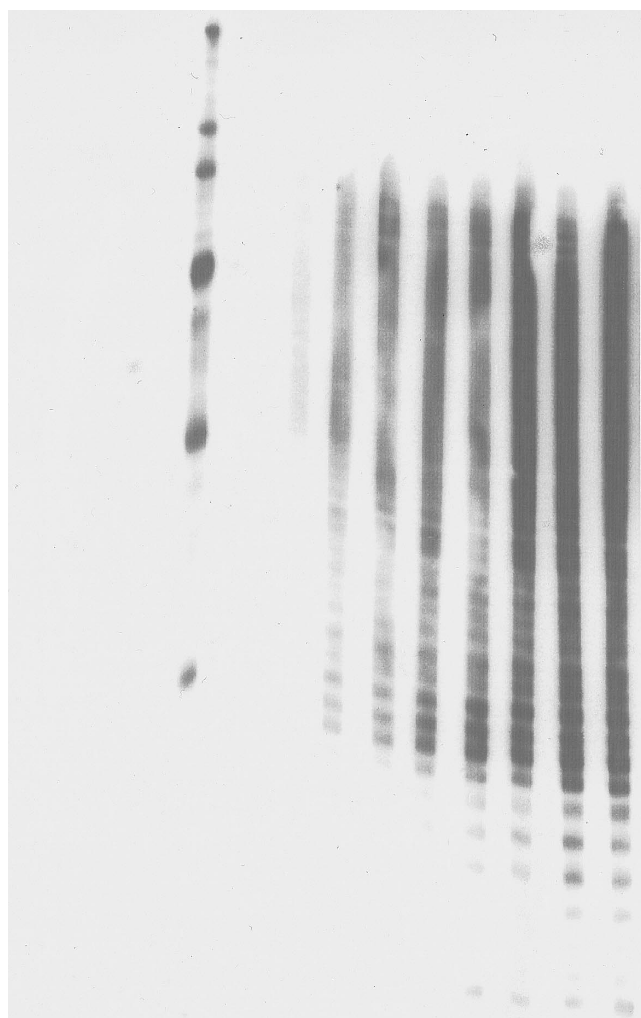

| Age (mo) | mw | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 33 | 36 |
|---|---|---|---|---|---|---|---|---|---|---|
| Immunoblot | | - | + | + | + | + | + | + | + | + |
| exoA | | <100 | <100 | 200 | 200 | 200 | 200 | 200 | 400 | 800 |
| OP cx | | + | + | + | + | + | - | + | + | - |
| BALF cx | | - | | | | + | | | | + |

**Figure 3.** Comparison of serology and culture results for a representative patient (004), demonstrating a difference in the timing of conversion to positivity. Reactivity on immunoblot to whole bacterial proteins is demonstrated on the gel, below which are shown positivity (+) or negativity (−) of immunoblot, exotoxin A (exoA) ELISA (reciprocal of dilution), and culture (cx) results for *Pseudomonas aeruginosa* on oropharynx (OP) and bronchoalveolar lavage fluid (BALF) specimens at the noted age, in months (mo). mw, Molecular-weight marker.

for by differences in patient population: well infants identified by neonatal screening versus infants with a clinical diagnosis of CF before entry into the study, patient age, indications for BAL, and the definition of infection. It would be expected that the current study design, examining children diagnosed on the basis of clinical criteria and following them up longitudinally for ⩽3 years, might bias the data toward a higher prevalence of *P. aeruginosa* infection.

Despite being considered the reference standard, even BALF culture may not reflect what is going on in the CF lung. Regional sampling of the lung may not accurately predict what organisms are present in other areas. Additionally, bacteria adherent to mucous plugs may not be sampled in BALF cultures.

Although the frequency of positive culture results paralleled those reported in the literature, when evidence of *P. aeruginosa* infection was based on a combination of culture and *P. aeruginosa* serology, the percentage of infected infants increased to 97.5%, greatly exceeding previously reported rates. Serologic studies predicted a higher incidence of infection than did OP or BALF cultures. This is not unexpected, for a number of reasons. First, a positive culture requires the presence of viable organisms. It has been suggested that, early in CF, initial colonization with nonmucoid *P. aeruginosa* can be eradicated [19], unlike colonization in advanced disease, in which eradication is virtually never seen. Second, the immune response to the organism, as evidenced by antibody production, may reflect past or current infection. In fact, "infection" is frequently distinguished from "colonization" on the basis of the host immune response. Bacterial isolation from a site such as the lung, which is not normally sterile, does not necessarily indicate infection, without evidence of an antibody response to the presumed infecting organism [20]. Evidence of an acute inflammatory response (either local or systemic) is also helpful in defining infection, although this may be more difficult in CF, because of the generally increased inflammation [21]. Finally, positive serology may reflect initial *P. aeruginosa* infection at a nonrespiratory site. Although other investigators [22] have not identified gastrointestinal colonization as a source of *P. aeruginosa* infection in CF, the current study was not designed to look at this.

Serologic tests reflecting *P. aeruginosa* infection in CF have been found to correlate with chronic infection [23–27]. Specific antibodies against exotoxin A and phospholipase C are elevated earlier in infection than are antibodies against alkaline protease and elastase [25]. However, none of these studies was conducted with young infants or with patients who might have been acquiring *P. aeruginosa* for the first time.

Antibodies against *P. aeruginosa* whole-cell proteins have been less well investigated, because immunoblot assays are a cumbersome screening tool for CF lung infection. Hancock et al. [28] demonstrated a correlation between ELISA and immunoblot results, using outer membrane protein antigens for both. However, only chronically colonized patients had these antibodies. The discrepancy between previous results and those of the current study may reflect several important differences. The most critical of these is the use of each patient's own isolates as the source of bacterial antigens. Previous studies have used laboratory strains rather than clinical isolates and have

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used outer membrane antigens rather than whole-cell proteins. Because many CF isolates have a mucoid phenotype, this may mask the outer membrane proteins. The use of whole-cell proteins allows the examination of exoproducts, as well as intrinsic membrane proteins, and may result in the earlier detection of an immune response. Additionally, because the immunoblot identifies antibodies to whole-cell proteins rather than just a single *P. aeruginosa* product, it is likely to be more sensitive.

The current study confirms the finding of genotypic variability in CF isolates of *P. aeruginosa* [29]. Each patient was infected with unique genotypes, which suggests that there is not a single clone of *P. aeruginosa* that initially colonizes young patients. There was no evidence of cross-infection among children at the 3 centers examined. Farrell et al. [30] recently reported an increased incidence of *P. aeruginosa* in young children with contact with other CF patients, but they did not perform genotyping, to determine whether they were the same clone. The majority of patients had a single genotype initially isolated that did not persist throughout the study. When new genotypes were acquired, it was sequential or intermittent. Occasionally, patients had multiple genotypes from the same culture. An important finding was that the genotype from the upper airway culture accurately predicted that in the lower airway.

The use of a single OP culture as a surrogate marker for lower airway colonization, in a subset of this patient population, has been reported elsewhere [5]. The sensitivity, specificity, PPV, and NPV of a single concurrent OP culture were of similar magnitude in the 2 studies. However, the PPV and NPV of 2 OP cultures are a significant improvement, which suggests that 2 upper airway cultures 3 months apart, with specimens for culture collected as described, could be a useful surrogate to help predict lower airway colonization in nonexpectorating children.

The early isolates identified in the current study (nonmucoid and antibiotic susceptible) were phenotypically distinct from those usually seen late in CF. "Classic" CF isolates are most often mucoid and may be nonpigmented [31, 32]. However, most isolates from young infants in this study were nonmucoid, and most had green pigmentation, suggesting pyoverdin and/or pyocyanin production. In 2 different populations of CF patients, ~80% of isolates were mucoid [33, 34]. However, in a study of non-CF isolates, only 3% of *P. aeruginosa* respiratory tract isolates had a mucoid phenotype [34]. This is similar to the findings of the current study, in which 2% of lower airway and 14% of upper airway isolates were mucoid. This supports the hypothesis advanced by Speert et al. [12] that *P. aeruginosa* convert to the mucoid phenotype under environmental pressure in the airway of patients with CF. However, our investigation of potential stimuli for phenotypic or genotypic changes identified no temporal association with antibiotic treatment or serologic response. The other finding unique to these early isolates of *P. aeruginosa* was the high percentage of isolates from both upper and lower airways that were susceptible to all antibiotics tested. CF isolates from patients with more advanced disease are often highly antibiotic resistant [31], which is thought to result from the frequent use of antibiotics in this population. However, most patients in the current study (95%) received intravenous or inhaled antibiotics during the study period, suggesting that multiple courses of antimicrobials may be required to affect resistance.

The serologic results presented herein suggest that infection with *P. aeruginosa* may begin much earlier than was reported in previous studies that were based on culture detection of organisms. Because these early isolates are generally nonmucoid and antibiotic susceptible, they are potentially easier to treat. Thus, this may represent a unique window for intervention to eradicate the organism before the onset of chronic infection, with its accompanying inflammatory response. It will be important to better understand the pressures that lead to changes in the genotype and phenotype of *P. aeruginosa* in CF, because this may also affect our ability to eradicate infection. Further studies of the utility of the serologic response in the diagnosis and monitoring of *P. aeruginosa* infection in CF appear to be warranted at this time and may, ultimately, further our understanding of the pathogenesis of the lung inflammation that is seen early in CF.

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