The role of serum *Pseudomonas aeruginosa* antibodies in the diagnosis and follow-up of cystic fibrosis

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In cystic fibrosis (CF), if *Pseudomonas aeruginosa* (Pa) infection is not diagnosed and treated early, chronic colonization occurs, which causes rapid decline in pulmonary functions. The aim of this study was to evaluate Pa antibodies, compare them with Pa cultures and determine their role in early diagnosis and follow-up. Ninety CF patients were included; they were divided into chronic, intermittent, negative, and mucoid groups. They were evaluated every 3-6 months. In each visit, pulmonary function tests and sputum cultures were obtained, and Pa antibodies exotoxin A (ExoA), elastase (ELA) and alkaline protease (AP) were determined in the serum by enzyme-linked immunosorbent assay (ELISA). The most specific test that discriminated chronic colonized patients from noncolonized patients was Pa culture, and the presence of at least one antibody had the highest sensitivity. AP had the highest specificity, and ELA had the highest sensitivity. All antibodies were highest in the mucoid group. ELA was highest in chronic and lowest in the negative group. The presence of antibodies was much higher than positive Pa cultures in patients younger than five years of age. A negative correlation between forced expiratory volume in 1 second (FEV1) and AP was determined only in the mucoid group. In the two-year follow-up, antibody presence did not show a regular pattern. In CF, Pa antibodies can be early markers for diagnosis, especially in young children who cannot expectorate, but they should only be used together with sputum cultures for long-term follow-up and treatment.

Key words: alkaline protease, cystic fibrosis, elastase, exotoxin A, Pseudomonas aeruginosa antibodies.

In cystic fibrosis (CF), pulmonary infection with *Pseudomonas aeruginosa* (Pa) causes clinical deterioration, and if not diagnosed and treated with early antibiotic treatment, it causes chronic colonization, which leads to severe lung damage. Pa infection can be diagnosed by sputum cultures or oropharyngeal swabs or bronchoalveolar lavage (BAL) cultures. BAL culture is currently accepted as the "gold standard" technique for obtaining lower airway pathogens in young children[1]; however, it is not routinely used, as it is an invasive method. Sputum cultures are difficult to obtain in preschool children and infants, as they are not generally able to expectorate, and upper airway cultures are known to have poor predictive accuracy for lower airway pathogens[1,2]. Therefore, additional markers are needed to diagnose Pa infection, and evaluation of antibodies against Pa may be helpful.

The aims of this study were to evaluate three major serum Pa antibodies -- exotoxin A (ExoA), elastase (ELA) and alkaline protease (AP) -- in CF patients, to compare them with sputum Pa cultures and to determine their role in early diagnosis and longitudinal follow-up.

A section of this study was presented at the ERS 21st Annual Congress, Amsterdam, the Netherlands, 24-28 September 2011.

Material and Methods

Patients

Cystic fibrosis (CF) patients diagnosed with two elevated sweat chloride levels and/or mutation analysis and who were being followed in our hospital were included over a period of two years between March 2008 and 2010. They were divided into four groups according to their sputum or oropharyngeal or BAL cultures in the last year. The "chronic" group was defined as patients with chronic Pa colonization who had at least three positive Pa cultures in the previous one year. The "intermittent" group was defined as patients with intermittent Pa colonization who had at least two positive Pa cultures in the last year but at least one negative Pa culture in between. The "negative" group included CF patients with no Pa growth in their respiratory cultures. The "mucoid" group was defined as patients with chronic mucoid Pa colonization who had at least three positive mucoid Pa cultures in the last year. All patients were evaluated every 3-6 months, and they had four clinical visits in total. At every visit, sputum or oropharyngeal cultures and pulmonary function tests were obtained and 3-4 ml blood was drawn. Patients continued their routine treatment, but patients who were using systemic corticosteroids were excluded. The study was approved by the local ethics committee.

Serum Pa Antibodies

Serum antibodies for three purified Pa antigens -- ExoA, ELA and AP -- were evaluated with Mediagnost, Germany®, which uses the enzyme-linked immunosorbent assay (ELISA) system. Results were reported as positive and negative.

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) 11.0® program was used for statistical analysis. The Kruskal-Wallis test was used for comparison of demographic values of patients, the chi-square test for comparison of ExoA, ELA and AP positivity between groups, the McNemar test and univariate analysis for sensitivity, specificity, and positive and negative predictive values, and the Point bi-serial correlation test for evaluation of the correlation between Pa antibodies and forced expiratory volume in 1 second (FEV1) and age and FEV1.

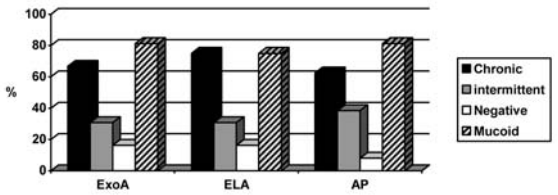

Figure 1. Positive antibody results in the first visit.

Results

Ninety patients in total were included. Their ages ranged between 8 months and 26.3 years (median age: 10.9 ± 7 years). The female/male ratio was 1:1. Only 48 patients could perform pulmonary function tests. Their FEV1 values were between 20% and 124%, and median FEV1 was 70% ± 21.7. There were 24 (26.7%) patients in the chronic group, 13 (14.4%) in the intermittent group, 37 (41.1%) in the negative group, and 16 (17.8%) in the mucoid group (Table I). The median ages were statistically different between groups (p: 0.037), but median FEV1 values were not statistically significant (p>0.05) (Kruskal-Wallis test).

The first visit

In the first visit, positive values for ExoA, ELA and AP were 66.7% , 75% and 62.5% in the chronic group, 30.8%, 30.8% and 38.5% in the intermittent group, 16.2%, 16.2% and 8.1% in the control group, and 81.3%, 75% and 81.3% in the mucoid group, respectively (Fig. 1). The differences between groups were statistically different (p<0.001) (chi-square test). When the chronic and mucoid groups were combined, those numbers were 72.5%, 75% and 70% for ExoA, ELA and AP, respectively.

Posivite Pa cultures were highest in the mucoid

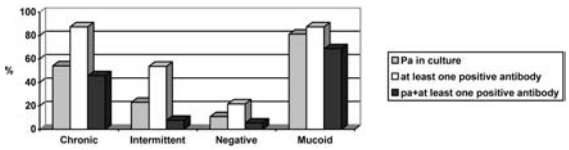

Figure 2. Culture Pa positivity and at least one antibody presence in the 4 groups in the first visit.

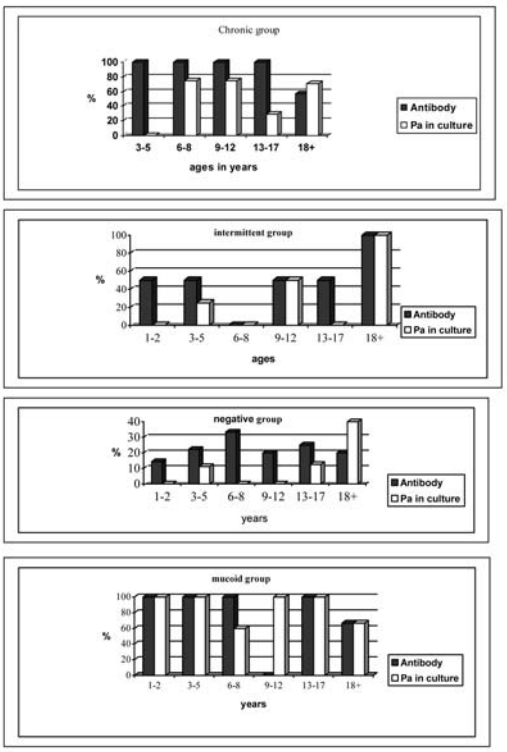

Figure 3. At least one positive antibody and Pa in the culture according to ages in each group in the first visit.

group (81.3%), and highest antibody titers were in both the chronic and mucoid groups (87.5% in both groups). Both culture positivity and at least one positive antibody were highest in the mucoid group (68.8%) (Fig. 2).

In patients younger than 5 years, at least one antibody positivity was higher than Pa culture positivity in all groups, and this difference was very obvious in the chronic group (Fig. 3).

The highest specificity to discriminate patients chronically colonized with mucoid and non-mucoid Pa (chronic + mucoid groups) from patients not chronically colonized with Pa (intermittent + negative group) was culture Pa positivity (86%); presence of at least one positive antibody had the highest sensitivity (100%); ExoA, ELA and AP positivity had the highest positive predictive value (100%); and presence of at least one antibody had the highest negative predictive value (87.5%) (Table II).

Both in all groups and individually in each group, there was no statistical difference in FEV1 percentage values between patients with negative and positive antibodies, for each antibody (Point bi-serial correlation). However, a negative correlation between FEV1 and AP positivity was determined only in the mucoid group. In this group, median FEV1 percentage was 85% in patients with negative AP antibodies, whereas it was 59% in the positive AP group (Point bi-serial correlation). There was no correlation between ages and antibody positivity in any of the groups, but there was a negative correlation between age and ELA only in the chronic group (54%, p: 0.006) (Point bi-serial correlation).

Follow-up visits

The number of patients evaluated in the second visit was 22 (91.7%), 12 (92.3%), 37 (100%), 14 (87.5%); in the third visit was 20 (83.3%), 10 (76.9%), 33 (89.2%), 13 (81.3%); and in the fourth visit was 18 (75%), 8 (61.5%), 24 (64.9%), 11 (68.8%) in the chronic, intermittent, negative, and mucoid groups, respectively.

In the two-year follow-up period, the antibody positivity had fluctuations and had no regular or similar pattern in all patients. In addition, there was no regular pattern in culture Pa positivity and serological results in any of the groups.

Table I. Patient Characteristics

	Chronic	Intermittent	Negative	Mucoid
Patient number n (%)	24 (26.7)	13 (14.4)	37 (41.1)	16 (17.8)
Age range*	36 m – 26.3 y	9 m- 18.5 y	8 m - 25.3 y	9 m – 25 y
Median age (months) (± SD)	173.5 (83.6)	144 (78.6)	90 (84.8)	125 (80.5)
Male/female	12/12	7/6	20 /17	6 /10
FEV1 range (%)	35 - 124	51 - 107	49 - 98	20 - 118
Median FEV1 (± SD)	69 (22.7)	77 (21.2)	68.5 (17.3)	64 (26.2)

*m: Months. y: Years. FEV1: Forced expiratory volume in 1 second.

Table II. Predictive Values to Discriminate Chronic Infected from Nonchronic Patients in the First Visit

	Specificity (%)	Sensitivity (%)	Positive predictive value (%)	Negative predictive value (%)
Pa in the culture	86	65	78.8	75.4
ExoA	80	43.3	100	78.4
ELA	80	44.4	100	80
AP	84	40	100	77.8
At least one positive antibody	70	87.5	70	87.5

Pa: *Pseudomonas aeruginosa*. ExoA: Exotoxin A. ELA: Elastase. AP: Alkaline protease.

Patients in the intermittent and negative groups who had no Pa in their cultures in the beginning but had positive Pa cultures in the follow-up visits ("culture conversion") and their serological results are shown in Table III. Among those patients, in the intermittent group, 3 patients had antibody positivity before culture conversion and 1 patient remained serologically negative. In the negative group, 1 patient had positive serology before culture conversion, 1 patient had positive serology after culture conversion and 3 patients remained serologically negative. *Staphylococcus aureus* was grown in the cultures of 3 patients in the intermittent group (Case numbers 109, 14, 26) and 4 patients in the negative group (Case numbers 42, 102, 108, 4).

P. aeruginosa (Pa) never grew in 6 and 28 patients in the intermittent and negative groups, respectively. Among those patients, at least one antibody was positive all through the study period in 4 and 8 patients in the intermittent and control groups, respectively (Table IV). *S. aureus* was grown in the cultures of 1 patient in the intermittent group (Case number 128) and 4 patients in the control group (Case numbers 43, 23, 28, 30).

In the chronic and mucoid groups, among patients who had no Pa culture in some visits and had positive cultures in other visits, all had positive antibodies in every visit except 5 patients in the chronic and 1 patient in the mucoid group who had negative serology (Table V). In the chronic and mucoid groups, among patients who had Pa growth in cultures at every visit, all had positive antibodies at every visit as well. At least one antibody was positive in visits in which there was no growth of Pa in the culture in all patients in the mucoid group and all patients except one in the chronic group.

Table III. Patients in Intermittent and Negative Groups with Culture Conversion and Serologic Results

Case number	Pa1	ab1	Pa2	ab2	Pa3	ab3	Pa4	ab4
Intermittent								
109	0	+	0	+	0	+	1	+
14	0	+	1	+	1	+	1	+
26	0	-	1	-	0	-	1	-
57	0	+	0	+	0	+	1	+
Negative								
119	0	+	1	+				
42	0	-	0	-	1	-		
102	0	-	1	-	0	-	1	+
108	0	-	1	-	0	-	0	-
4	0	-	1	-	0	-	0	

Pa1,2,3,4: *Pseudomonas aeruginosa* in the culture in the first, second, third and fourth visit, respectively (1: Pa grown in the culture, 0: Pa not grown in the culture).
ab 1,2,3,4: At least one antibody in the first, second, third and fourth visit, respectively (+: positive, -: negative).

Table IV. Patients in Intermittent and Negative Groups Who Never Had Pa in the Culture But Had at Least One Positive Antibody

Case number	Pa1	ab1	Pa2	ab2	Pa3	ab3	Pa4	ab4
Intermittent								
127	0	+						
129	0	-	0	-				
128	0	-	0	+		+		
8	0	+	0	+	0	-		
123	0	+	0	+	0	+		
9	0	-	0	-	0	-	0	-
Negative								
101	0	-	0	-				
111	0	-	0	-	0	-		
7	0	-	0	-	0	-		
11	0	-	0	-	0	-	0	-
12	0	-	0	-	0	-	0	-
15	0	-	0	-		-	0	-
32	0	-	0	-	0	-		
33	0	-	0	-				
38	0	-	0	-	0	-		
43	0	+	0	+	0	+		
58	0	-	0	-	0	-	0	-
106	0	-	0	-	0	-	0	-
115	0	-	0	-	0	-		
121	0	-	0	-	0	-	0	-
20	0	-	0	-	0	-	0	-
21	0	-	0	-	0	+	0	-
23	0	+	0	-	0	-	0	-
24	0	-	0	-	0	-		
27	0	-	0	-	0	-	0	-
28	0	+	0	-	0	-	0	-
30	0	+	0	+	0	+	0	-
31	0	+	0	+	0	+	0	+
45	0	-	0	-	0	-	0	-
46	0	-	0	-	0	-	0	-
59	0	-	0	-	0	-	0	-
60	0	-	0	-	0	-	0	-
117	0	-	0	-	0	+	0	+
125	0	-	0	+	0	+	0	+

Pa1,2,3,4: *Pseudomonas aeruginosa* in the culture in the first, second, third and fourth visit, respectively (1: Pa grown in the culture, 0: Pa not grown in the culture).
ab 1,2,3,4: At least one antibody in the first, second, third and fourth visit, respectively (+: positive, -: negative).

Discussion

In this study, three major serum Pa antibodies in four CF patient groups were evaluated and compared with their sputum Pa cultures over a two-year period.

Since an ELISA method was introduced for serum Pa antibodies, the relationship between acquisition of Pa infection and formation of antibodies is still being investigated, but there has not been much or clear evidence. In recent years, although a correlation between cultures and antibodies was evaluated, it can

be challenging to interpret individual responses in each patient.

In previous studies, it was suggested that rising serum antibody titers to Pa antigens might have occured before detection of Pa in the culture of respiratory secretions[3-5] and antibody responses were also high in chronic infection in addition to early infection[3,5]. However, some studies failed to demonstrate positive Pa antibodies in early infection[6,7]. These different results may be due to different methods as well as different cut-off levels. The sensitivity and specificity of Pa antibodies as well as their cut-off values are still not adequately studied.

P. aeruginosa (Pa) antibody formation can be affected by many factors like the immunological status of the patient, corticosteroid use, antibiotic treatment, and different Pa phenotypes[8]. It has been postulated that they are increased in severe CF disease and might have proinflammatory effects[8]. Mucoid Pa strains were shown to produce more pronounced antibody responses than nonmucoid strains[4,9]. Dual colonization with *S. aureus* infection was demonstrated to decrease ExoA and ELA levels and to increase phospholipase C levels[10].

In this study, we showed that the most specific marker in the culture was Pa and the marker with highest sensitivity was the presence of at least one antibody for discriminating patients

Table V. Serological Results in Patients in the Chronic and Mucoid Groups Who Had No Pa in the Cultures at Any Visit

Case number	Pa1	ab1	Pa2	ab2	Pa3	ab3	Pa4	ab4
Chronic								
40	1	+	1	+	0	+	1	+
3	1	-	0	+	1	+	1	+
29	1	+	0	+	1	+	1	+
49	1	+	0	+	1	-	1	-
112	0	+	1	+				
114	0	+	1	+	1	+		
120	0	+	1	+				
104	0	+	1	+	1	+	1	+
105	0	+	1	+	1	+	1	+
116	0	-	1	+	0	+	0	+
22	0	+	0	+	1	+	1	-
44	0	+	1	+	1	+	0	+
52	0	+	1	+	0	+	1	-
53	0	+	0	+	1	+	1	+
63	0	+	0	+	1	+	0	+
Mucoid								
131	1	+						
48	1	+	0	+				
110	1	+	0	+	1	+		
17	1	+	0	+	1	+	1	+
2	1	+	1	+	0	+	0	+
19	1	-	1	+	0	+	1	+
51	1	+	0	+	0	+	1	+
39	0	+						
47	0	+	0	+	1	+	0	
50	0	+	1	+	1	+	1	+

Pa1,2,3,4: Pa in the culture in the first, second, third and fourth visit, respectively (1: Pa grown in the culture, 0: Pa not grown in the culture).

ab 1,2,3,4: At least one antibody in the first, second, third and fourth visit, respectively (+: positive, -: negative).

chronically colonized with Pa from patients not chronically colonized. This means that at least one positive antibody shows a high probability of Pa infection. AP had the lowest sensitivity but the highest specificity, which was in accordance with the literature[8], and which indicates that evaluating AP antibodies would be useful in excluding Pa colonization. In our study, ELA had the highest sensitivity, but it was found to have higher sensitivity in another study[8]. In addition, positive predictive values of 100% for each antibody in our study suggest that their presence might be valuable for determining the presence of Pa colonization.

All three antibodies were highest in the mucoid group, followed by the chronic, intermittent and negative groups. It has been reported in previous reports that antibodies can be positive in the intermittently colonized group, and this could be a clue for infection[5,11]. In our study, it was surprising to find positive Pa antibodies in the negative group in whom Pa was never cultured in their respiratory cultures; however, this may be explained by the fact that Pa is a microorganism living in the natural environment and even healthy people can be infected with this microorganism. In a previous study, Pa antibodies were demonstrated in healthy people[11].

It has been suggested that Pa antibodies rise in a regular order; ExoA rises in the beginning, followed by ELA as the disease progresses, but ELA is decreased when Pa becomes mucoid, as the composition of lipopolysaccharide changes[3,9]. Therefore, it was suggested that ELA response could be predictive of a more advanced infection[12]. Our results support these data, as ELA was highest in the chronic group but lowest in the negative group.

In our study, in all four groups, the presence of at least one antibody was higher than Pa in the culture, which shows the importance of searching for antibodies for diagnosing infection. Especially in children younger than five years, the presence of at least one antibody in the serum was higher than the presence of Pa in the cultures, especially in the chronic group. In the study by Tramper-Stranders et al[8], serological tests were found to have no additional value for diagnosing Pa colonization in children aged 4-6 years, and in another study by Douglas et al[1], serum Pa antibodies were evaluated and compared to BAL cultures and found to have only limited value for detecting early Pa infection in infants and preschool children; nevertheless, a recent study evaluating Pa antibody titers in young children with CF diagnosed through newborn screening showed that regular serologic assessment of Pa antibodies in these children can be a worthy diagnostic tool if age-specific serological cut-off values are used rather than fixed values[13]. Our data support these results, although we were not able to study Pa titers and the results were reported as positive or negative.

We could not demonstrate a correlation between antibody positivity and FEV1 values except in the mucoid group. This may mean that the severity of the disease can be monitored by the presence or the rise in AP antibodies in patients who are chronically colonized with mucoid Pa strains. In one study, an inverse correlation was demonstrated between Pa antibody titers and forced vital capacity (FVC), FEV1, and forced expiratory flow $(FEF)_{25-75}$ in chronically colonized patients, and it was suggested that an exaggerated immune response to Pa was associated with pulmonary damage in CF[14].

In contrast to our cross-sectional results suggesting that Pa antibodies may have a potential value in diagnosing Pa infection/colonization, we could not show their potential to be beneficial in longitudinal follow-up. We demonstrated that they had no regular pattern and had fluctuations independent of their culture results in each group. Similar results were shown in a cohort study in which there was no relation between increasing antibody titers and chronic Pa colonization, and the height of the antibody titers could not predict whether early colonization would become chronic[8]. The antibody results are difficult to interpret in CF patients as they may be affected by different strains of Pa, the presence of other microorganisms and acute pulmonary exacerbation. The positive antibody results in the longitudinal follow-up of the intermittent and negative groups may be due to false-positive ELISA results, but it should be kept in mind that the sputum or oropharyngeal cultures were obtained every 3-6 months in our study, and in this interval, there might have been a transient Pa infection and the Pa microorganism could have been missed in

the cultures. Therefore, Pa infection cannot be excluded in patients with negative cultures but positive antibodies. Furthermore, in our study, it was not recorded whether patients had acute pulmonary exacerbation or were using antibiotics at the time serum were drawn for antibody analysis, so the presence of acute pulmonary exacerbation or antibiotic use might have influenced their antibody results as well.

In the longitudinal follow-up of the chronic and mucoid groups, among patients who had no Pa growth in the culture in any visit but had Pa growth in other visits, the antibody responses were positive in all but five patients in all visits. Thus, in this group of patients, the serology alone cannot give information about the Pa status of the patient.

In conclusion, serum antipseudomonal antibodies can be an early sign of Pa infection or colonization in patients with CF, especially in young children who cannot expectorate. However, they should not be used alone in the follow-up of patients in order to define their infection or their response to treatment. Serum antipseudomonal antibodies should only be used in combination with respiratory cultures in both the diagnosis and follow-up of Pa infection/chronic colonization.

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