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Pseudomonas aeruginosa serology and risk for re-isolation in the EPIC trial☆

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Abstract

Background—The prognostic value of *Pseudomonas aeruginosa* serology for antibiotic therapy in cystic fibrosis patients is not well understood.

Methods—Using five antigens from two ELISAs, we assessed whether positive serology in CF patients participating in the multi-center Early *Pseudomonas* Infection in Children (EPIC) trial would predict treatment failure, time to pulmonary exacerbation and risk for recurrent *P. aeruginosa* isolation post eradication.

Results—Baseline positive *P. aeruginosa* serology was not significantly associated with failure of initial *P. aeruginosa* eradication measured at week 10 (adjusted for baseline culture) but seropositivity to the antigens alkaline protease and exotoxin A was significantly associated with increased risk for recurrent *P. aeruginosa* isolation during the 60 week post eradication follow-up

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Appendix A. Supplementary data

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period (p=0.003 and p=0.001 respectively). There was no association between baseline seropositivity and time to pulmonary exacerbation.

Conclusion—*P. aeruginosa* serology may complement culture results in clinicians' efforts to successfully monitor recurrence of early P. aeruginosa in CF patients.

Keywords

Pseudomonas; Serology; Treatment; Infection; EPIC

1. Introduction

Pseudomonas (P.) aeruginosa remains the major pathogen in patients with cystic fibrosis (CF). However, studies using several antibiotic regimens have demonstrated that eradication of the pathogen from the patients' airways is possible when therapy is initiated in the acute phase of infection early after isolation. This strategy has been associated with considerably delaying the onset of chronic pulmonary *P. aeruginosa* infection in CF patients, decreasing the progression of lung disease, and reducing the cost of antibiotic intervention [1, 2]. A prerequisite for applying this therapeutic strategy is close monitoring of *P. aeruginosa* isolation in CF subjects based on the assumption that the therapeutic window is small and the switch of non-mucoid *P. aeruginosa* to biofilm formation would render eradication of the pathogen difficult or impossible [3]. Detection is generally accomplished by, oropharyngeal (OP) cough swabs, induced sputum or bronchoalveolar lavage (BAL) specimens [4, 5]. In the US, the standard of care for detection of initial *P. aeruginosa* infection in pre-expectorating patients is to collect routine OP cultures at least every 3 months [6]; Furthermore, while OP cultures are easy to perform on a frequent basis, they appear to only moderately reflect lower airway infection [7]. Serological tests have been used to diagnose *P. aeruginosa*, particularly in patients who do not produce sputum (reviewed in [3, 8]). However, this method has not been unequivocally accepted due in part to differences in patient population, study design, and cut-off antibody titers among various studies [9–12].

P. aeruginosa serology has been demonstrated to be an adjunct method for assessment of early eradication therapy [2, 8, 13], because bacterial culturing, particularly from nasopharyngeal aspirates and OP cough swab specimens, is subject to sampling error, and imperfect diagnostic accuracy [7]. Highly sensitive ELISAs for detection of serum antibodies against *P. aeruginosa* antigens in immunocompetent CF patients may complement less than perfect surveillance methods such as OP swabs. Several assay systems have been developed, for different *P. aeruginosa* antigens such as *P. aeruginosa* whole cell proteins and exotoxin A [14, 15], exotoxin A, elastase and alkaline protease [2, 8, 13, 16], and components of the type III secretion system [17, 18].

Elevated antibodies among young subjects at the time of initial *P. aeruginosa* positive OP culture may be a useful tool for CF clinicians and researchers monitoring patients at risk for subsequent infection. Here we demonstrate the relationship between culture and serologic data available in subjects with newly acquired *P. aeruginosa* infection participating in the multi-center Early *Pseudomonas* Infection in Children (EPIC) trial [19] and determine if

baseline *P. aeruginosa* serology would predict treatment failure, time to pulmonary exacerbation and risk for re-isolation of *P. aeruginosa* post eradication.

2. Methods

2.1. Subjects and serologic analysis

Subjects with CF, recruited for the EPIC Clinical trial, were 1–12 years of age and had *P. aeruginosa* isolated within six months prior to study entry [19]. The study consisted of an initial 10 week treatment period (in an attempt to eradicate *P. aeruginosa*) plus 60 weeks of follow up. Serum specimens, collected during the EPIC trial at weeks 0, 22, 46 and 70, were used in the present study to assess antibody titers against *P. aeruginosa*. Data from up to eight respiratory cultures were available from weeks 0, 3, 10, 22, 34, 46, 58 and 70, to compare serological data to standard of care OP culture results. For serology, the E-15 ELISA kit (Mediagnost, Reutlingen, Germany) tests for antibodies against the *P. aeruginosa* antigens alkaline protease, elastase, and exotoxin A [8, 16] was used. Additionally, different ELISAs were used, developed at the Medical College of Wisconsin (MCW) (Milwaukee, Wisconsin, USA), to include the pooled *P. aeruginosa* antigens PopB and ExoS, PAO1 cell lysate preparation [17], and exotoxin A to compare and calibrate to the E-15 ELISA. The technical personnel who carried out the ELISAs were blinded to CF subject data in the EPIC trial. Positive and negative antibody titers were evaluated according to instructions of the manufacturer with regard to the E-15 ELISA, while sero-positivity using the MCW ELISA was defined by antibody titers exceeding a cut-off value of 100. This threshold was selected for all antigens based on earlier results and Receiver Operating Curve (ROC) analysis of pilot data [20].

2.2. Statistical analysis

Individual antigen titers were summarized and plotted by concurrent culture status. Concordance, defined as the proportion of samples that were both sero-positive and culture positive or sero-negative and culture negative, and diagnostic properties, including sensitivity, specificity, positive predictive value, and negative predictive value, were calculated for the E-15 and MCW combined ELISAs (if any one antigen in the ELISA is positive, the combined ELISA is positive) and for each antigen individually. *P. aeruginosa* positivity was plotted over time for the E-15 and MCW ELISAs in addition to culture. Modified Leeds criteria were used to group subjects according to *P. aeruginosa* culture positivity over the course of the 70 week study [21]: *P. aeruginosa* free, intermittent *P. aeruginosa* (at least one positive but less than half of the subject's cultures) or chronic *P. aeruginosa* (50% or more of a subject's cultures are *P. aeruginosa* positive). Antigen titers were plotted as a function of these categories. Odds ratio and 95% confidence intervals were calculated to evaluate the relationship between sero-positivity at baseline, week 0, and at eradication of the pathogen at week 10 (unable to isolate with OP swab culture). In subjects who were culture negative at week 10, time to next *P. aeruginosa* isolation, determined by culture, was analyzed as a function of seropositivity at baseline using time-to-event analyses and the log-rank test. Likewise, time to pulmonary exacerbation was analyzed as a function of baseline sero-positivity. Analyses were performed using R 2.11.1 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

There were 304 CF subjects in the EPIC trial [19]. From 303 subjects, serum and culture data were available for *P. aeruginosa* serology analysis, resulting in 975 paired serum/culture specimens over the four time points at weeks 0, 22, 46 and 70 (see Supplement Fig. 1). Among those with baseline serum, 238 (92.9%) had week 10 presence or absence of *P. aeruginosa* by culture to determine the success of antibiotic treatment in eradicating the pathogen (unable to isolate with OP swab culture). Baseline characteristics of the entire subject cohort and the week 10 *P. aeruginosa* culture status have been reported elsewhere [19, 22].

3.1. P. aeruginosa serology and culture data

To investigate whether serological results from the individual antigens used in the ELISAs would correlate with the *P. aeruginosa* culture status of the CF subject, ELISA results for each antigen were pooled over all time points and compared with the *P. aeruginosa* culture status. In an attempt to maximize the detection of serum antibodies directed against *P. aeruginosa*, we used in our analysis a broad panel of *P. aeruginosa* antigens, including the extracellular toxins alkaline protease, elastase, exotoxin A and exotoxin S, the pore protein PopB and a PAO1 cell lysate preparation. Sera from CF subjects who were *P. aeruginosa* culture-positive revealed higher antibody titers than sera from *P. aeruginosa* culture-negative CF subjects (Fig. 1). The spread of titer values was highest in E-15 ELISA alkaline protease, elastase, and exotoxin A, while the distribution of titer values in the MCW antigens was narrower.

To assess whether eradication of *P. aeruginosa* from OP swabs during the EPIC trial period, would be reflected by a similar loss of sero-positivity, results of the E-15 and MCW ELISAs were plotted over the investigation period and compared with data from *P. aeruginosa* cultures. While *P. aeruginosa* culture positivity sharply declined from 40% at baseline to approximately 12% at subsequent visits in accordance with antibiotic treatment, antibody titers against *P. aeruginosa* antigens used in the E-15 and MCW ELISAs declined only slightly from 63% and 47% at baseline to 52% and 38% at week 70, respectively (Fig. 2).

Next we assessed whether the ELISAs might be used to differentiate between *P. aeruginosa* free, intermittent *P. aeruginosa*, and chronic *P. aeruginosa*, as defined by the modified Leeds criteria [21]. Participants who were chronically *P. aeruginosa* culture positive had generally higher baseline serology than those who were only intermittently culture positive and those who never turned *P. aeruginosa* culture positive by the end of the study (Fig. 3). Persistence of high titers over the course of the study was observed in individuals with chronic *P. aeruginosa* infection (Supplement Fig. 2), and at week 70, titers from chronically *P. aeruginosa* culture positive subjects were significantly higher than those who remained free of *P. aeruginosa* for all 6 antibodies against their antigens (p-values < 0.01).

Pooled concordance across the four time points ranged from 0.60 to 0.79 between the individual antigens and respiratory *P. aeruginosa* culture results (Table 1). Among the discordant pairs, serum antibody titers were more often positive than *P. aeruginosa* cultures with regard to the E-15 antigens alkaline protease (144/253), elastase (169/264), exotoxin A

(328/393) and the MCW antigens PopB+ExoS (130/232) and PAO1 cell lysate (217/296). Assuming the respiratory *P. aeruginosa* culture as standard of care, the sensitivities for the individual antigens were low, ranging from 0.26 to 0.67. In comparison, specificity of the individual antigens was relatively high, ranging from 0.58 to 0.99, indicating that a large fraction of samples had titers less than the reciprocal cut-off titer of 100 among culture negative events. The positive predictive values for the single ELISA antigens were low (<0.5), whereas the negative predictive values were moderately high (0.83–0.88). Combining antigens, as exemplified in the E-15 ELISA improved the sensitivity (E-15: 0.78; MCW: 0.69) while decreasing the specificity (E-15: 0.48; MCW: 0.69).

3.2. Predictive utility of P. aeruginosa serology

Next we sought to know whether a positive antibody titer against one or several of the *P. aeruginosa* antigens at baseline in CF subjects was associated with eradication or time to next positive *P. aeruginosa* culture after successful eradication. Eradication rate of *P. aeruginosa* at week 10 among those with baseline serology was 207/238 (86.9%). Boxplots of baseline antibody titers for each of the antigens by eradication status at week 10 (Supplement Fig. 3) indicated that there were some slightly increased baseline titers in those who failed to eradicate at week 10, but there was an overlap in the serology values between the two groups and thus not statistically significant. Baseline serology (seropositive or seronegative) in the combined E-15 ELISA was not found to have a significant relationship with initial eradication success after adjusting for baseline culture status (OR=1.97, 95% CI=[0.75, 5.19], p-value=0.17). Similarly, the combined MCW ELISA for baseline serology was not significantly associated with initial eradication after adjusting for baseline culture status (OR=1.2, 95% CI=[0.53, 2.73], p-value=0.66).

Using a Cox Proportional Hazard model, we demonstrate that positive baseline antibody titers against the E-15 ELISA antigens alkaline protease and exotoxin A were both significantly associated with a higher risk of *P. aeruginosa* reisolation in the 60 weeks after eradication (HR=2.20, 95% CI= [1.30, 3.71], p-value =0.003) and (HR= 2.50, 95% CI= [1.42.4.40], p-value= 0.001), respectively (Table 2). Seropositivity at baseline for all other ELISA antigens and the combined ELISAs were found to increase the risk of *P. aeruginosa* recurrence but this finding was not statistically significant.

A similar question was whether a positive antibody titer against one or several of the *P. aeruginosa* antigens at baseline in CF subjects in whom *P. aeruginosa* was successfully eradicated would be associated with a shorter time to next acute pulmonary exacerbation; the primary clinical outcome of the EPIC trial. Results based on the E-15 ELISA revealed that sero-positive CF subjects did not have a different risk for pulmonary exacerbation requiring antibiotics or hospitalization than those who were sero-negative at baseline (CI=1.1, 95% CI=[0.77, 1.57], p-value=0.61).

4. Discussion

Our study of serology in the EPIC trial examines the utility of blood antigen detection as a prognostic marker of subsequent OP culture isolation after initial eradication of Pa from upper respiratory tract swabs. In addition, the use of serology as a prognostic marker of key

clinical outcomes such as pulmonary exacerbations was evaluated. We report that elevation of 2 antigens, exo A and alkaline protease, prior to eradication therapy in CF subjects in whom *P. aeruginosa* was successfully eradicated, was significantly associated with a higher risk of *P. aeruginosa* re-isolation during the 60 week follow-up period. Serology was not predictive, however, of pulmonary exacerbation during this time period. As displayed in Fig. 2, it is evident that OP swabs and Pa serology have different time courses in response to early Pa infection. Antibody response against *P. aeruginosa* was high initially, and serum antibody titers remained elevated over the course of the study period, while *P. aeruginosa* culture positivity dropped significantly at the end of the first treatment course It is therefore not surprising that only a moderate rate of concordance was observed between serology and culture collected at the same time,. It is well established [7, 9] that OP culture is by no means a perfect diagnostic indicator for lower or upper airway *P. aeruginosa* infection; however in the US it is the standard of care [6]. The very fact that serology does not exactly mimic culture results, highlights the complementary potential of *P. aeruginosa* serology in CF.

The diagnostic and concordance results alone make it impossible to determine if serology characterizes residual *Pa* infection, precursor to infection, or if the discordance is a product of the limitations of OP. Host response to initial *P. aeruginosa* infection appears to be variable in cystic fibrosis. Our results confer that early *P. aeruginosa* infection does not always elicit a serologic response [9], and conversely, serology often perpetuates when respiratory culture is *Pa* negative—the latter being more often the case. The sustained elevation of serology when OP culture positivity appears to wane may be due to slow disappearance of *P. aeruginosa*-positive plasma cells after the eradication of the antigenic stimulus in the organism or due to persistence of *P. aeruginosa* antigens in obstructed airways of CF subjects despite negative *P. aeruginosa* culture results.

In previous studies [2, 8, 13] a *P. aeruginosa* ELISA had been used to monitor the success of the antibiotic intervention in CF subjects for eradication of early *P. aeruginosa* infection based upon the return of elevated serum antibodies toward normal levels. We could not confirm these findings in the present study as there was no statistically significant association between baseline serology and eradication rate at week 10 after adjusting for baseline culture status. We note above, however, that positive antibody titers *against* 2 antigens, alkaline protease and exotoxin A, were significantly associated with a higher risk of *P. aeruginosa* re-isolation. These data suggest that previous *P. aeruginosa* isolation may predispose a CF patient to a subsequent *P. aeruginosa* isolation episode in a shorter time period. Thus, CF patients with new acquisition of *P. aeruginosa* and positive serology may benefit from closer monitoring (by culture or culture-independent techniques [23–25]) than those with negative serology, enhancing the ability to quickly treat recurrent infection. Because serology appears to contain information not captured by OP culture, or BAL [9] culture, we hypothesize that a combination of detection methods may yield better diagnosis and prognosis than does each independently [23–25]. The design of the EPIC study did not provide the ideal setting to answer questions about optimal surveillance algorithms using OP and serology in combination. Rather, the results compel the CF community to consider evaluation of potential protocols through prospective trials — which may include serology,

OP, BAL, sputum, or polymerase chain reaction genomic identification methods. Two and three stage screening programs, where no one diagnostic tool is sufficient, can provide effective monitoring [26, 27]. The combination of screening tools in other disease settings such as cervical [28, 29] and prostate [30, 31] cancer yields accurate disease detection. Only prospective trials comparing different surveillance algorithms will confirm efficacy and effectiveness on an individual level.

Since all subjects in the EPIC clinical trial had a new or recent *P. aeruginosa* positive respiratory culture and the time period of the trial was relatively short, a longitudinal analysis of the correlation between the time period of *P. aeruginosa* culture positivity and serological data in CF subjects was not possible. Serologic data from the observational cohort of the EPIC study are currently being analyzed and will provide further insights into the longitudinal relationship between *P. aeruginosa* serology and *P. aeruginosa* culture results.

From a pathophysiological standpoint, it is still unclear, how the findings in the sero-positive CF cohort with higher risk for earlier *P. aeruginosa* isolation could be interpreted. Possibly, this group may have previously had a higher number of *P. aeruginosa* isolations, a higher bacterial burden in the airways, and as a consequence, more lung tissue damage than those CF patients with sero-negative baseline findings. The notion that specific antibodies against *P. aeruginosa* antigens augment lung inflammation and tissue damage due to immune complex mediated type III reactions, resulting in worse long term prognosis, has been repeatedly demonstrated in the past [32–35].

Ultimately patient experience and clinical endpoints determine the usefulness of any diagnostic tool or monitoring algorithm. There was no statistically significant association between baseline serology with pulmonary exacerbation requiring antibiotics or hospitalization over 60 weeks of follow-up; however our findings and those from other investigators would suggest that *P. aeruginosa* serology may have diagnostic and prognostic value to CF clinicians in their efforts to early detect re-isolation of *P. aeruginosa*. Prospective, randomized trials of monitoring algorithms inclusive of serology, and culture, and culture independent methods are needed to evaluate effectiveness of *P. aeruginosa* monitoring protocols on clinical outcomes in CF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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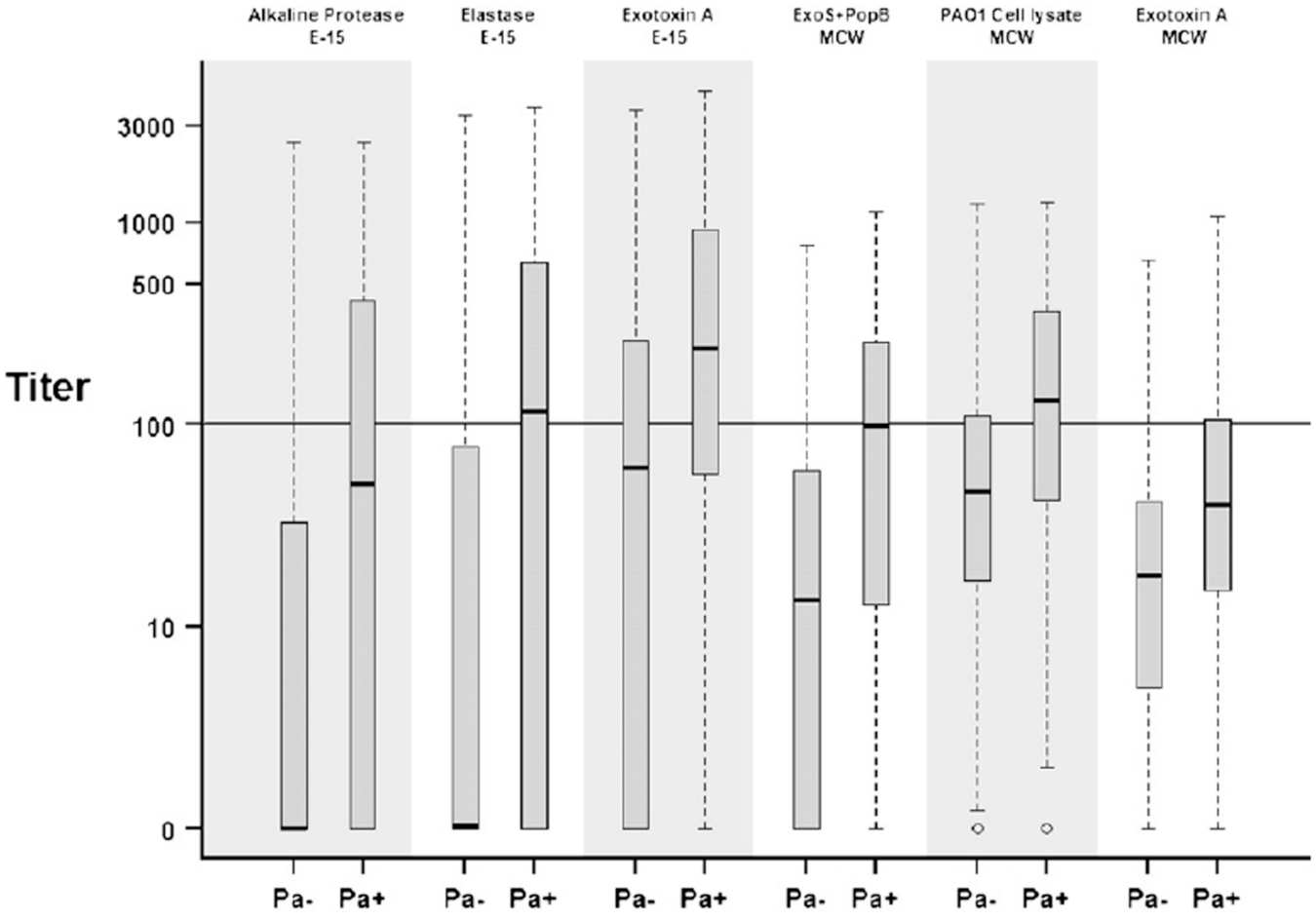

Fig. 1.

Distribution of individual antigen titers by concurrent culture status for all 975 serum samples. Pa+ indicates *P. aeruginosa* positive, Pa− indicates *P. aeruginosa* negative. Hash lines are the median values, boxes represent the 25th and 75th percentiles (inter-quartile range), whiskers extend to minimum and maximum or 1.5× the inter-quartile range (whichever is smaller), outliers are marked as circles.

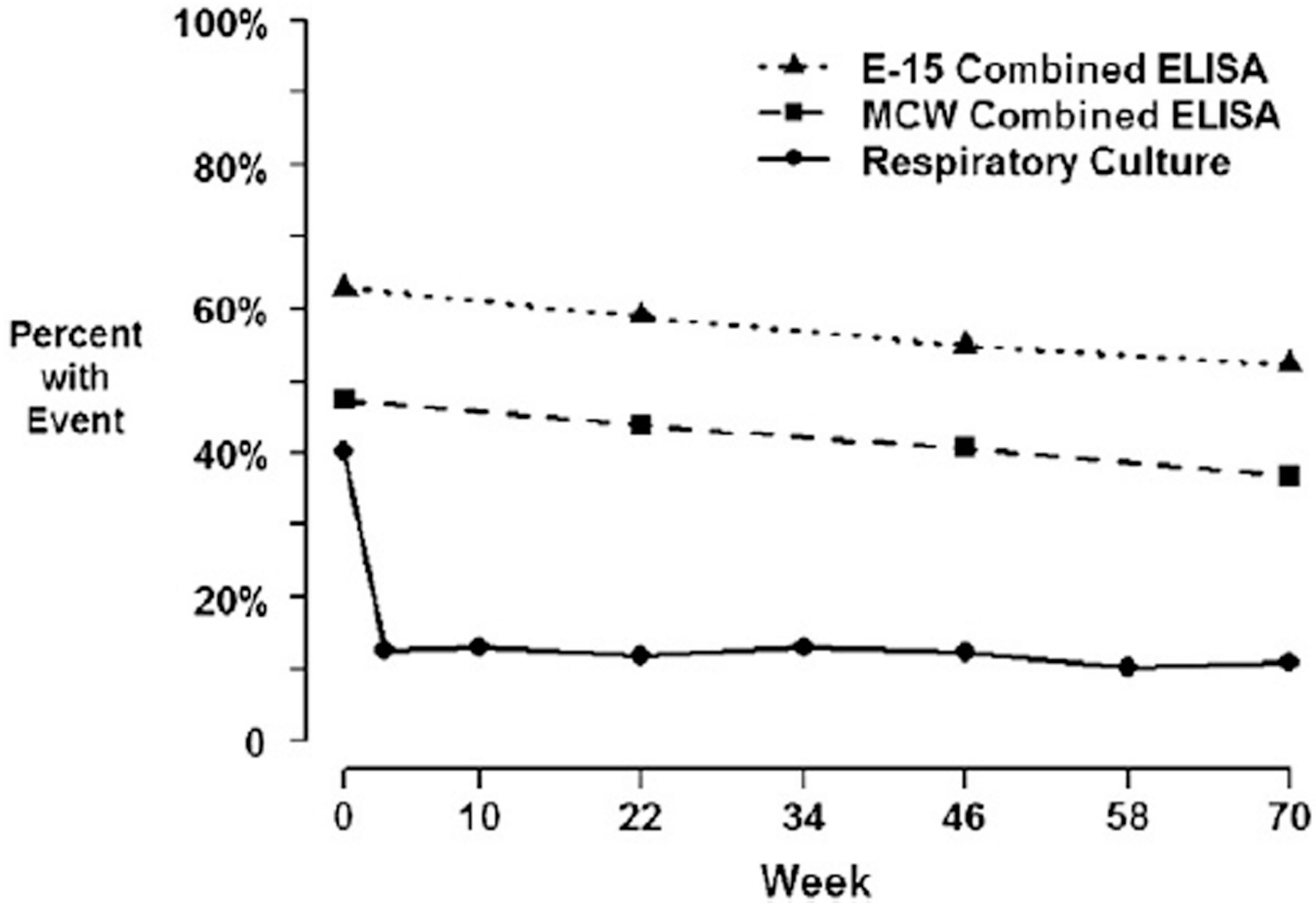

Fig. 2.

Culture positivity and seropositivity over time. The solid black line shows the proportion culture positive for *P. aeruginosa* across each visit among the 303 subjects with available serology. The dashed lines show the proportion of samples seropositive at each visit for each combined ELISA. A combined ELISA is defined as seropositive if any one of the three antigens in an ELISA has titer at least 100.

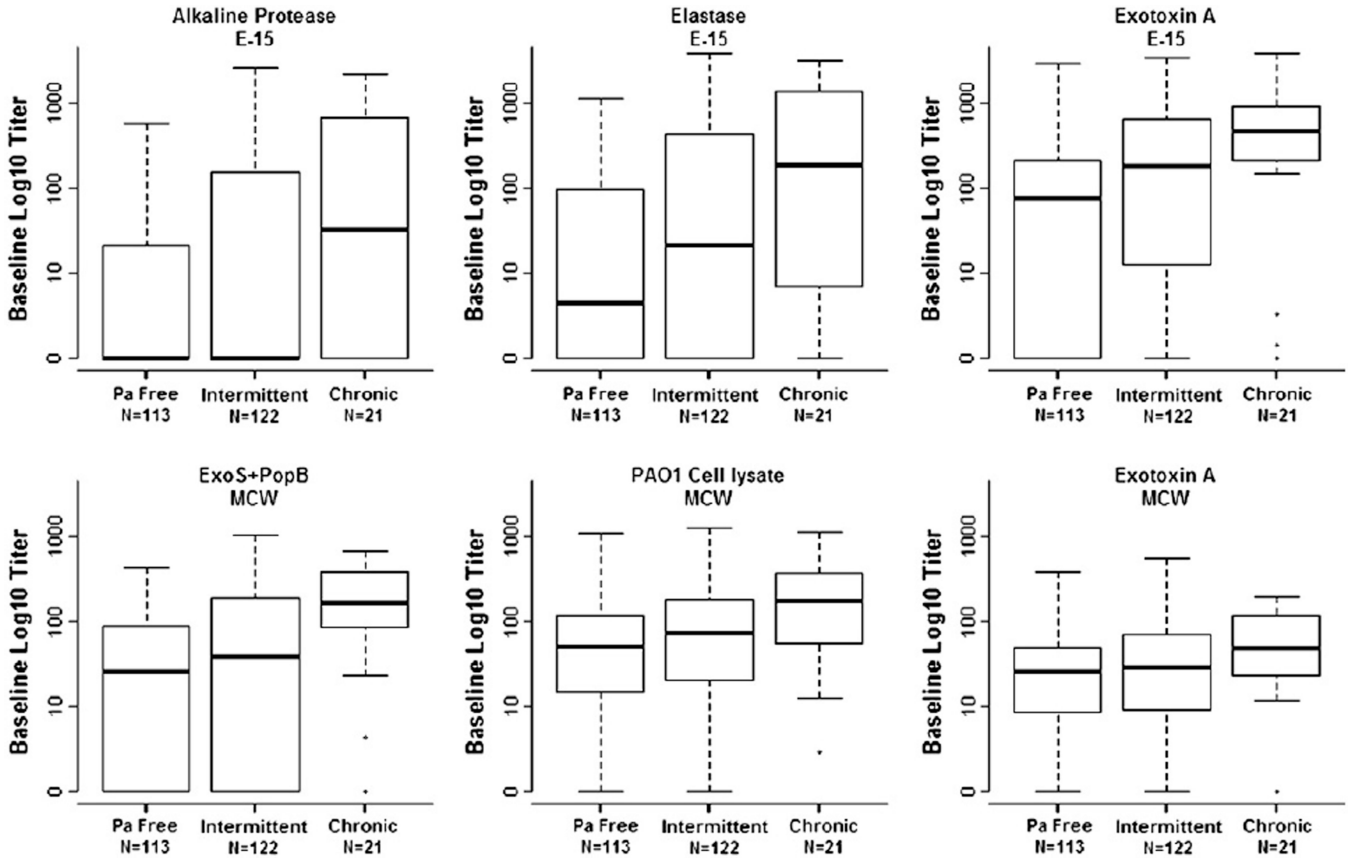

Fig. 3.

Baseline serology titers by respiratory culture status using modified Leeds criteria. Subjects were classified as '*Pa* free' if they were clear of *P. aeruginosa* from baseline through end of study, 'Intermittent' if they had less than half of all available cultures testing positive for *P. aeruginosa*, and 'Chronic' if they had half or more of all available cultures testing positive for *P. aeruginosa*. Hash lines are the median values, boxes represent the 25th and 75th percentiles (inter-quartile range), whiskers extend to minimum and maximum or 1.5× the inter-quartile range (whichever is smaller), outliers are marked as circles.

Table 1

Frequency and diagnostic properties of individual and combined ELISAs.

P.aeruginosa Antigen		Culture −	Culture +	Sensitivity	Specificity	PPV [a]	NPV [b]	Concordance [c]
Alkaline protease [d]	−	633	109	0.45	0.81	0.38	0.85	0.74
	+	144	89					
Elastase [d]	−	608	95	0.52	0.78	0.38	0.86	0.73
	+	169	103					
Exotoxin A [d]	−	449	65	0.67	0.58	0.29	0.87	0.60
	+	328	133					
PopB+ExoS [e]	−	647	102	0.48	0.83	0.42	0.86	0.76
	+	130	96					
PAO1 cell lysate [e]	−	560	79	0.60	0.72	0.35	0.88	0.70
	+	217	119					
Exotoxin A [a]	−	716	146	0.26	0.92	0.46	0.83	0.79
	+	61	52					
E-15 combined [d]	−	371	44	0.78	0.48	0.28	0.89	0.54
	+	406	154					
MCW combined [e]	−	502	61	0.69	0.65	0.33	0.89	0.66
	+	275	137					

[a] PPV refers to positive predictive value.

[b] NPV refers to negative predictive value.

[c] Concordance is the proportion of all 975 samples that were culture negative/seronegative or culture positive/seropositive.

[d] Component of Mediagnost® E-15 ELISA kit (Reutlingen, Germany).

[e] Studies conducted at Dr. Joseph Barbieri's laboratory, Medical College of Wisconsin.

Table 2

Hazard ratios for risk of *P. aeruginosa* occurrence post eradication treatment.

Antigen		Hazard ratio	Hazard ratio 95% CI	p-value
E-15	Alkaline protease	2.19	(1.30, 3.71)	0.003
	Elastase	1.66	(0.99, 2.78)	0.055
	Exotoxin A	2.50	(1.42, 4.39)	0.001
	Combined	1.68	(0.96, 2.95)	0.072
MCW	PopB+ExoS	1.51	(0.89, 2.56)	0.120
	PAO1 cell lysate	1.61	(0.97, 2.68)	0.068
	MCW exotoxin A	1.69	(0.89, 3.18)	0.106
	Combined	1.38	(0.83, 2.31)	0.219

Post-eradication is week 10 onward. Each hazard ratio is attributable to baseline seropositivity against the individual antigens, combined E-15 ELISA, and MCW ELISA. A combined ELISA is defined as seropositive if any one of the three antibodies in an ELISA has titer at least 100.