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Diagnostic value of serological tests against *Pseudomonas aeruginosa* in a large Cystic Fibrosis Population

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Abstract

Background

Serological methods to monitor *P. aeruginosa* colonisation in CF-patients are advocated, however the diagnostic value of a commercially available *P. aeruginosa* antibody tests to detect early and chronic *P. aeruginosa* colonisation in a non-research setting has not been assessed.

Methods

P. aeruginosa colonisation was estimated by regular culture of sputum or oropharyngeal swabs during 3 consecutive years in 220 CF-patients (0-65 years). Commercially available ELISA tests with 3 *P. aeruginosa* antigens (elastase, exotoxin A, alkaline protease) were performed at the end of the study period. In a subgroup of 57 patients (4-14 years) serological tests were performed annually.

Results

Using culture as reference standard, test characteristics of the ELISA tests calculated with the advised cut-off values showed a sensitivity of 79% and a specificity of 89% for chronic colonisation. ROC-curves were created to optimize cut-off values. Applying these new cut-off values resulted in a sensitivity of 96 % and a specificity of 79%. All 3 individual serological tests discriminated well between absence and presence of chronic *P. aeruginosa* colonisation. Sensitivity of the individual antibody test elastase was 87 %, exotoxin A 79 % and alkaline protease 76 %. First colonisation was preceded by positive serology in only 5 of 13 patients (38 %).

Conclusion

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

Introduction

Pulmonary colonisation with biofilm-producing strains of *P. aeruginosa* in cystic fibrosis (CF) patients is associated with decline in pulmonary function and subsequent morbidity and mortality.[1][2] In infancy, 10-30 % of patients are colonised with *P. aeruginosa*. This increases to 80-90% during adolescence.[3][4] Initial colonisation and early asymptomatic infection are close entities; these precede chronic colonisation and infection. The transition duration of initial asymptomatic colonisation and infection to chronic tissue-destroying infection and colonisation varies among patients. In this manuscript, we will therefore use the term colonisation. It is of major importance to detect *P. aeruginosa* at an early stage since aggressive treatment of early colonisation might postpone chronic colonisation.[5][6] Acquisition of *P. aeruginosa* is often monitored by culture of sputum or oropharyngeal swabs. Serological methods (Crossed Immune Electrophoresis (CIE), Western immunoblot, Enzyme Linked Immuno Assay (ELISA)) to detect *P. aeruginosa* are not routinely used in CF-centres.[3] [7][8] Precipitin measurement by CIE is largely taken over by ELISA and Western blotting techniques. CIE and whole cell protein ELISA have a high sensitivity (96%-100%) for chronic colonisation.[9] ELISA with purified antigens has a lower sensitivity (15%-100%), depending on the antigen and stage of colonisation.[10] Recently prospective studies suggested that antibodies may be present before the first positive culture.[11][12][13]

Antibody development is influenced by the immunological condition of the host, corticosteroid use, antibiotic treatment targeted against *P. aeruginosa* and *P. aeruginosa*-related factors like phenotype and production of exoproteins.[8] [10][11] Only scarce data are available on the clinical relevance of anti-*P. aeruginosa* antibody responses. Obviously, these antibodies fail to eliminate *P. aeruginosa* and lack protective effects.[14][15] The antibody response seems to be more prominent in patients with severe clinical disease, suggesting they might play a pro-inflammatory role.[16]

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

Methods

Participants

We performed the study in the CF-Centre Utrecht, the Netherlands. All patients with a diagnosis of CF, confirmed by sweat chloride test > 60 mmol/L and/or genotyping were eligible to participate in the study. The medical ethics committee of the University Medical Centre Utrecht approved the study. All participants or their parents gave written informed consent. Fifty two adults and 168 children (age < 18 years) participated in this cross-sectional study. Fifty-seven of the 168 children also participated in a 3-year prospective study.

Cultures

Sputum- or oropharyngeal cultures were carried out for all patients according to treatment protocol of the centre in three consecutive years from January 2002. When a patient was not able to produce sputum, an oropharyngeal swab was taken. The sensitivity and positive predictive value of oropharyngeal swabs range between 44 % and 83 %. However for the classification of colonisation status, negative predictive values [% of patients with a negative test (oropharyngeal culture) who do not have the disease (*P. aeruginosa* colonisation)] are more important. These negative predictive values range between 85 % and 97 %.[12] [17][18] Mean number of cultures per patient was 6 (median 4, range 3-43), depending on pulmonary condition.

Culture samples were inoculated onto blood- and McConkey agar. After incubation at 37°C, media were inspected for any growth of *P. aeruginosa* after 24 and 48 hours.

Classification of patients' *P. aeruginosa* colonisation status over the 3-year period was based on Lee's criteria [19] (chronic colonisation: > 50 % of all cultures positive; intermittent colonisation: ≤ 50 % of all cultures positive, with at least one positive culture; no colonisation: all cultures negative for *P. aeruginosa*).

ELISA

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

Serological testing was performed using a commercially available ELISA-kit (Mediagnost, Reutlingen Germany). This semi-quantitative IgG ELISA comprises of three common *P. aeruginosa* antigens, i.e. elastase, exotoxin A and alkaline protease. In brief, sera were diluted with a factor 10^3 with phosphate buffered saline. In each well of a 96-wells micro titre plate, 100 µl of a diluted sample was added and incubated at 37°C for two hours. After aspirating and washing 3 times intensively with phosphate buffered saline/Tween-20, 100 µl conjugate solution (anti-human IgG peroxidase) was added to each well. The plate was further incubated for 2 hours at 37°C. After washing thoroughly, wells were filled with 100 µl substrate solution (Tetramethyl Benzidine) and again incubated at room temperature, in dark. After half an hour, the reaction was stopped by sulphuric acid. Within 10 minutes, the optical density was read by a photometer set at 450 nm. Titres were extrapolated from optical density values of 2 negative and 2 positive control sera, according to the manufacturer's manual.

Lung function

Clinical data for every CF patient are collected in a database annually. Data collected at the end of the observation period in 2004 were used to compare lung function of chronic *P. aeruginosa* colonised patients with non-colonised patients [forced expiratory volume in 1 second as percentage of predicted (FEV1 % pred) and forced vital capacity as percentage of predicted (FVC % pred)].

Statistics

The ELISA manual advised a cut-off titre against one or more of the tested antigens of 1:500 or 1:1250 (borderline positive or positive respectively). When applying these cut-off titres to our study group, low sensitivities of the ELISA for chronic *P. aeruginosa* colonisation, with cultures as a reference standard, were found. Therefore, the ability of the serological tests to discriminate between chronic colonisation and no colonisation was estimated using the area under the receiver operating characteristic curve (ROC-area). Difference in discriminatory power between the individual serological tests was estimated by difference in ROC-area (ΔROC area) with 95% confidence intervals, taking into account the correlation between the expressions as they were based on the same cases. Cut-off titres with best discrimination (defined as the point on the ROC-curve closest to the upper left corner) were used to classify patients as having negative or positive serology.[20]

Lung function data of chronically colonised and non-colonised patients were compared using the Student's t–test, and multiple linear regression. These parameters were expressed as mean±SD. We considered differences significant if they exceeded the 0.05 probability level. Calculations were performed by Statistical Package for the Social Sciences (SPSS version 12.0, Chicago, Ill. USA).

Results

Participant characteristics

Mean age at the end of the observation period of the patients in the cross-sectional study was 14.5±10.6 years, in the prospective study 8.1±2.8 years. Chronic colonisation with *P. aeruginosa* was found in 67 patients, 60 patients were intermittently colonised and 93 patients were not colonised. Characteristics of the study population are summarized in table 1.

Table 1
Participant characteristics of cross-sectional and prospective study.

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

Impact of chronic *P. aeruginosa* colonisation on clinical characteristics was estimated. Mean FEV1 % pred and FVC % pred were significantly lower in chronically *P. aeruginosa* colonised patients compared with non-colonised patients (FEV1 % pred 64.8±29.2 versus 89.0±19.4 and FVC % pred 80.0±22.4 versus 94.1±13.4; $p < 0.001$, adjusted for age).

Diagnostic value of serological tests for chronic P. aeruginosa colonisation

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

The ROC-curves (figure 1) illustrate that all 3 different serological tests discriminate well between colonised and non-colonised patients. The ROC-area is highest for elastase (0.926), followed by alkaline protease (0.909) and exotoxin A (0.874). There were no significant differences in discriminatory power (ROC-area's) between the 3 serological tests. Test characteristics associated with best discriminatory cut-off titres are shown in table 2. Although alkaline protease has a higher ROC-area than exotoxin A, it has a somewhat lower sensitivity (79 % vs. 76%).

Table 2
ROC-area's and cut-off titre with test characteristics.

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

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

In chronic colonisation, 87 % of patients showed positive elastase, 79 % positive exotoxin A and 76 % positive alkaline protease serology. In the intermittently colonised group, we found positive serology in respectively 42% (elastase), 48% (exotoxin A) and 17% (alkaline protease) of patients. In the non colonised group, respectively 11 % (elastase), 19% (exotoxin A) and 3% (alkaline protease) of patients showed positive serology.

Combining results of individual serological tests, 96% of chronically colonised patients had at least one positive antibody titre. Elastase serology is the most sensitive test, but there were 6 elastase antibody-negative patients who appeared positive for other *P. aeruginosa* antibodies if a combination of antibody tests was used. Specificity of the combination of serological tests was 79%. Test characteristics calculated with the cut-off values for borderline positivity of the ELISA manual and test characteristics using the cut-off values listed in table 2 were compared: the sensitivity increased from 79% to 96%, while the specificity decreased from 89% to 79%.

Prevalence of P. aeruginosa positive cultures and positive serology

To measure additional value of serology in comparison with cultures to detect *P. aeruginosa*, data of cultures and serology from different age cohorts were analysed. We observed one or more *P. aeruginosa* positive cultures and/or positive serology during the 3-year study period in 20 % of youngest (0-2 y) patients, and in 81 % of the adult (>18 y) cohort (figure 2a). All paediatric age cohorts showed a higher number of patients with positive cultures than with positive serology. In the two youngest age cohorts (0-2 y and 3-5 y), no additional *P. aeruginosa* was detected by serology. From age cohort 6-8 years and older, evidence for additional *P. aeruginosa* colonisation was obtained by serology as compared to culture alone in 3-11 %. Chronic colonisation per age cohort is shown in figure 2b. None of the youngest patients (0-2 y) and 53 % of the adult cohort (>18 y) were chronically colonised with *P. aeruginosa*. Positive serology accompanied chronic colonisation in all age cohorts except for the 12-14 and 15-17 year cohort.

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

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

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

	2001		2002			2003				2004			2005	
	Pa*	Ela†	Exo	Alk	Pa	Ela	Exo	Alk	Pa	Ela	Exo	Alk	Pa	Pa
Pt.1	0	-	+	-	0	-	-	-	1	-	-	-	1	0
Pt.2	0	-	+	-	0	+	+	-	0	-	+	-	1	0
Pt.3	0	+	-	+	0	+	-	+	1				1	
Pt.4	0	-	-	+	0	-	-	-	0	+	+	+	1	1
Pt.5	0	+	-	-	0	-	-	-	1	+	+	+	1	1
Pt.6	0	-	-	-	0	-	-	-	0	-	-	-	1	1
Pt.7	0	-	-	-	0	-	-	-	1	-	-	-	0	0
Pt.8	0	-	-	-	0	-	-	-	1	-	-	-	0	0
Pt.9	0	-	-	-	0	-	-	-	1	-	-	-	0	0
Pt.10	0	-	-	-	0	-	+	-	1	+	+	-	1	1
Pt.11	0	-	-	-	1	-	-	-	0	-	-	-	0	0
Pt.12	0	-	-	-	1	-	-	-	0	-	-	-	0	1
Pt.13	0	-	-	-	0	-	-	-	1	-	-	-	0	0
Pt.14	0	+	-	-	1	-	-	-	1				0	1
Pt.15	0	+	+	+	1	+	+	-	0	+	+	-	0	0

*Pa=*P. aeruginosa* in culture, 0=no, 1=yes

†Ela=elastase; Exo=exotoxin A; Alk=alkaline protease; + = positive serology; - = negative serology

Discussion

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

Colonisation with *P. aeruginosa* is a clinically relevant issue. There is a strong impact of *P. aeruginosa* colonisation on lung function, even when adjusted for age. These data confirm previous studies that show worsening of clinical parameters after acquisition of *P. aeruginosa*.[2]

To detect *P. aeruginosa* colonisation, measurement of *P. aeruginosa* antibodies is advocated. Previous studies show high sensitivity and specificity of serological tests for chronic *P. aeruginosa* colonisation, depending on the serological method used.[9] [21][22] However, the some of these studies included very small numbers of patients and test characteristics can be influenced by different definitions of *P. aeruginosa* colonisation. Culture is often used as a reference standard. Culture sampling errors may occur and therefore may influence the test characteristics of serology. Therefore, in this study we examined cultures during 3 consecutive years to make a clear division between non-colonised and chronically colonised patients.

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

Elastase serology is the most sensitive test for chronic colonisation, with a sensitivity of 87%. Hollsing et al., using a different ELISA assay, found a sensitivity of elastase for chronic colonisation of only 23%, in a small study cohort.[10] Possible explanation of the large difference is a distinct definition of chronic colonisation. They made a classification of colonisation status based on cultures over a 6-month period prior to serology measurement, while we defined colonisation over a 3-year period. Another explanation might be the low cut-off titres that we used, but even with the cut-off titres advised by the manufacturer's manual, the sensitivity of our test was much higher. The sensitivity of the other two antibodies is also higher in our study in comparison with other studies. Exotoxin A has a sensitivity of 79%, but the specificity is lowest among all three antigens. The antibody alkaline protease has the lowest sensitivity (76%) but a very high specificity (97%) and might therefore be useful to rule out *P. aeruginosa* colonisation.

Single antigen ELISA's directed against *P. aeruginosa* virulence factors fail to show immunological response in all *P. aeruginosa* colonised patients. In our study, the detection rate increased from 87% to 96% when compared with single antibody testing with elastase.

Regarding successive cultures as reference standard, problems with definition of specificity may arise because early *P. aeruginosa* colonisation might not yet be proven by

positive cultures while there is positive serology. On the other hand, transient positive serology without positive cultures is also seen in our follow-up cohort. We did not determine antibodies in healthy controls, but as *P. aeruginosa* is a common pathogen from the environment, it is possible that contact with these bacteria leads to transient antibody formation. Healthy controls might have positive antibody titres, as was shown by Pedersen et al.[9]

In children between 4 and 6 years of age, serology was of no additional value to diagnose *P. aeruginosa* colonisation. Older patients have a higher prevalence of antibodies in absence of positive cultures. At ages 6-17 year, only a small fraction (3-11 %) of patients had positive serology without a positive culture. The prevalence of *P. aeruginosa* colonisation in the youngest patients was lower than described in recent literature. Burns showed that 72.5% of patients had evidence for *P. aeruginosa* colonisation by culture and 97.5% by both culture and serology before the age of 3 years. In the study of Li, almost 90% of 3-year old patients had acquired *P. aeruginosa* in their cultures.[4] [12]

The prospective data of 57 children show that in case of culture conversions to a positive culture, no uniform serology pattern is seen. From the thirteen patients that showed culture conversion, only five showed early positive serology that could have been helpful in diagnosis. Thus, serological methods with one or more individual antigens as a screening tool for early *P. aeruginosa* colonisation in a clinical setting does not seem to be very sensitive. The failure of serology to detect early colonisation in young patients empasises the need for continued reliance on culture. West et al. described also that only 54% of children showed positive exotoxin A serology before or at the time a culture became positive.[13] In our cross-sectional study, the antibody that was elevated most often in non colonised patients was exotoxin A, but in the longitudinal follow up, it was not clear that a rise in exotoxin A titres occurred before rise of other titres. The height of antibody titres could not predict early colonisation to become chronic. Possibly, the cohort was too small to find a relation between increasing antibody titres and chronic *P. aeruginosa* colonisation.

In majority of patients with positive serology and negative culture, antibodies disappeared. It seems that single positive antibody titres without concomitant positive cultures do not predict whether a patient will become colonised.

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

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Competing interests:
None

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

Legends to the figures

Figure 1: ROC-curves of 3 individual serological tests.

Figure 2a: Prevalence of *P. aeruginosa* positive cultures and positive serology per age cohort.

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

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Figure 1
ROC-curves of 3 individual serological tests.

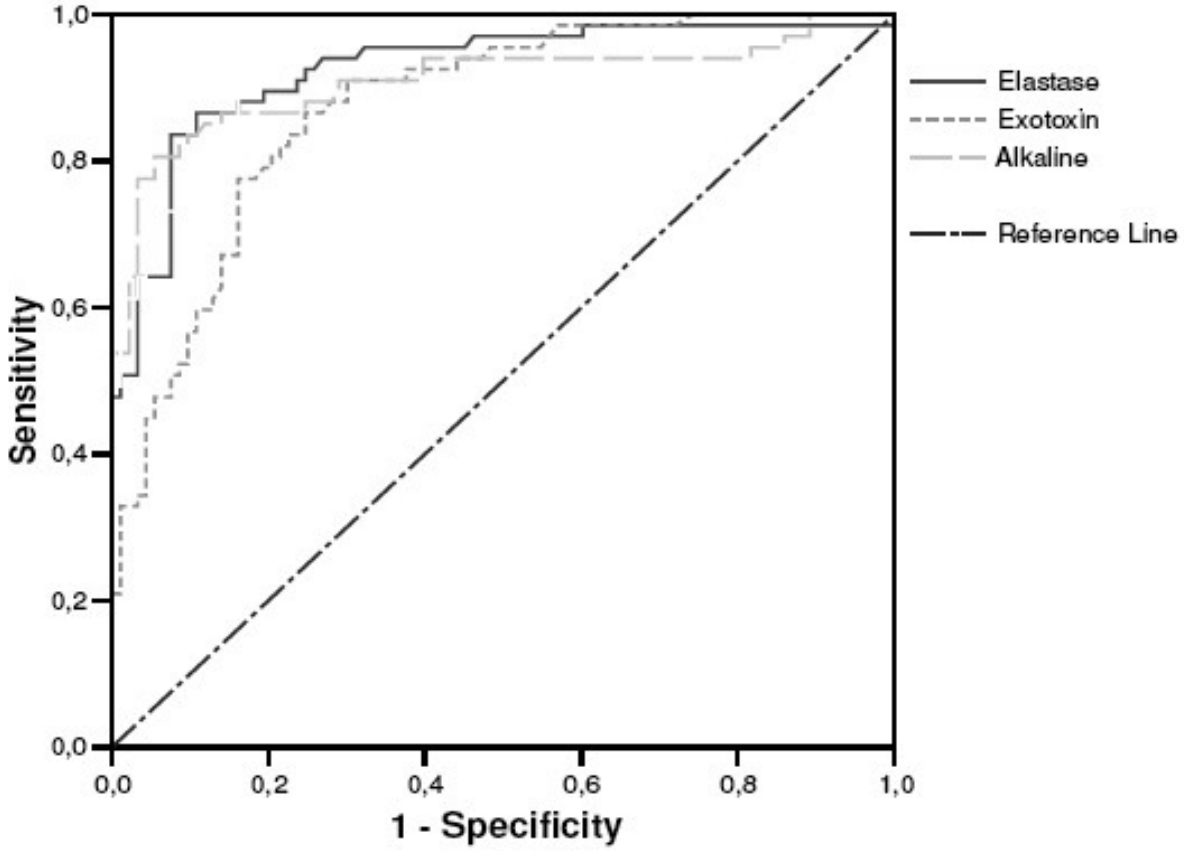
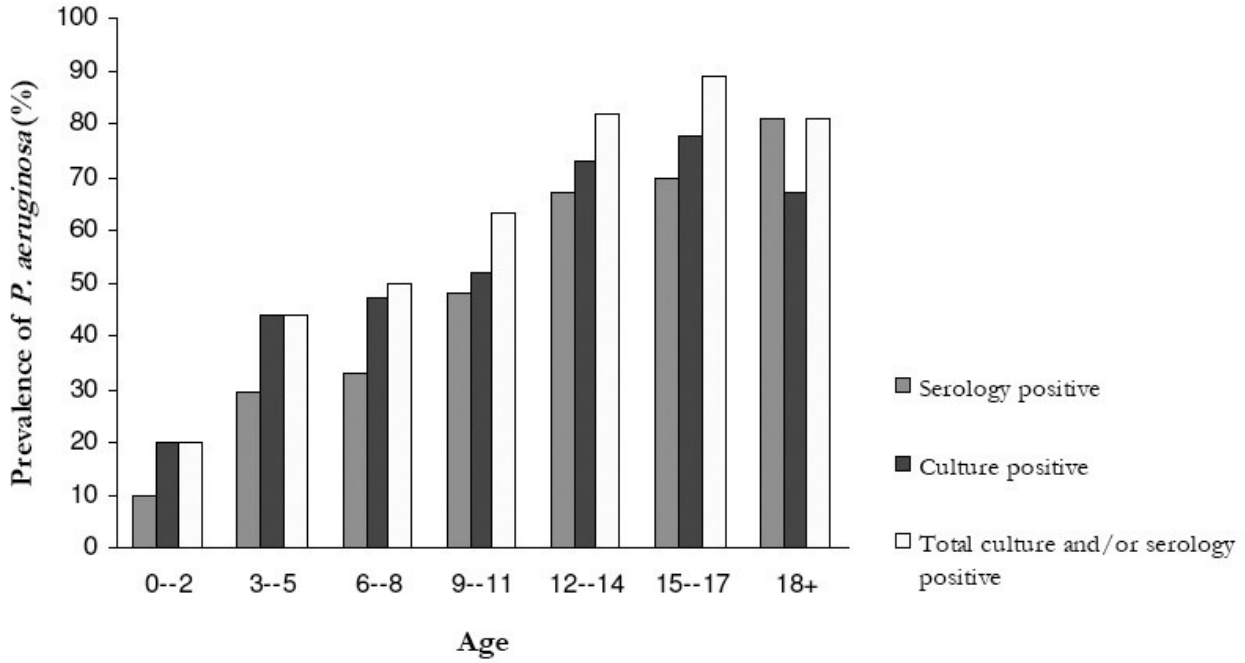


Figure 2a
Prevalence of *P. aeruginosa* positive cultures and positive serology* per age cohort.



*positive serology: one or more of the serological tests positive

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