Diagnostic value of serological tests against *Pseudomonas aeruginosa* in a large cystic fibrosis population

G A Tramper-Stranders, C K van der Ent, M G Sliker, S W J Terheggen-Lagro, F Teding van Berkhout, J L L Kimpen, T F W Wolfs

**Background:** Serological methods to monitor *Pseudomonas aeruginosa* colonisation in patients with cystic fibrosis (CF) are advocated but the diagnostic value of a commercially available *P aeruginosa* antibody test to detect early and chronic *P aeruginosa* colonisation in a non-research setting has not been assessed. **Methods:** Colonisation with *P aeruginosa* was estimated by regular culture of sputum or oropharyngeal swabs during three consecutive years in 220 patients with CF aged 0–65 years. Commercially available ELISA tests with three *P aeruginosa* antigens (elastase, exotoxin A, alkaline protease) were performed at the end of the study period. In a subgroup of 57 patients (aged 4–14 years) serological tests were performed annually. **Results:** Using culture as the reference standard, the ELISA tests using the advised cut off values had a sensitivity of 79% and a specificity of 89% for chronic colonisation. Receiver-operator characteristic curves were created to optimise cut off values. Applying these new cut off values resulted in a sensitivity of 96% and a specificity of 79%. All three individual serological tests discriminated well between the absence and presence of chronic *P aeruginosa* colonisation. The sensitivity of the individual antibody test was 87% for elastase, 79% for exotoxin A, and 76% for alkaline protease. First colonisation was preceded by positive serological results in only five of 13 patients (38%). **Conclusion:** In patients with CF, serological tests using specific antigens are sensitive for diagnosing chronic *P aeruginosa* colonisation. However, the failure of serological tests to detect early colonisation in young patients emphasises the need for continued reliance on cultures.

Pulmonary colonisation with biofilm producing strains of *Pseudomonas aeruginosa* in patients with cystic fibrosis (CF) is associated with a decline in pulmonary function and subsequent morbidity and mortality. In infancy 10–30% of patients are colonised with *P aeruginosa*, and this increases to 80–90% during adolescence. 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 paper 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 delay or prevent chronic colonisation. Acquisition of *P aeruginosa* is often monitored by culture of sputum or oropharyngeal swabs. Serological methods such as crossed immune electrophoresis (CIE), Western immunoblot, and enzyme linked immunoassay (ELISA) to detect *P aeruginosa* are not routinely used in CF centres. Precipitin measurement by CIE has been largely taken over by ELISA and Western blotting techniques. CIE and whole cell protein ELISA have a high sensitivity (96–100%) for chronic colonisation. ELISA with purified antigens has a lower sensitivity (15–100%), depending on the antigen and stage of colonisation. Recent prospective studies suggested that antibodies may be present before the first positive culture.

Antibody development is influenced by the immunological condition of the host, corticosteroid use, antibiotic treatment targeted against *P aeruginosa*, and *P aeruginosa* related factors such as phenotype and production of exoproteins. Only a few 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. The antibody response seems to be more prominent in patients with severe clinical disease, suggesting they might play a pro-inflammatory role. In this study we assessed the value of a commercially available serological test using three purified antigens to detect chronic *P aeruginosa* colonisation in a large CF population. We also examined the value of these serological tests for early detection of *P aeruginosa* colonisation.

**METHODS**

**Participants**
The study was performed in the CF Centre, Utrecht, The Netherlands. All patients with a diagnosis of CF, confirmed by a sweat chloride test >60 mmol/l and/or genotyping were eligible to participate in the study. Fifty two adults and 168 children (age <18 years) participated in the cross sectional study; 57 of the 168 children also participated in a 3 year prospective study.

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

**Cultures**
Sputum or oropharyngeal cultures were carried out for all patients according to the treatment protocol of the centre in

**Abbreviations:** CF, cystic fibrosis; ELISA, enzyme linked immunoassay; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity
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 ranges from 44% to 83%. However, for the classification of colonisation status, negative predictive values (percentage 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%.2 17 The mean number of cultures per patient was six (median 4, range 3–43), depending on pulmonary condition.

Culture samples were inoculated onto blood and McConkey agar plates. After incubation at 37°C, media were inspected for any growth of P. aeruginosa. After 24 and 48 hours. Classification of P. aeruginosa colonisation status of patients over the 3 year period was based on the criteria of Lee et al19 (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).

Enzyme linked immunosassay (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 serum samples were stored at −20°C. Serological testing was performed using a commercially available ELISA kit (Mediagnost, Reutlingen, Germany). This semi-quantitative IgG ELISA consists of three common P. aeruginosa antigens: elastase, exotoxin A, and alkaline protease. In brief, serum samples were diluted by a factor of 102 with phosphate buffered saline (PBS). In each well of a 96-well microtitre plate, 100 μl of a diluted sample was added and incubated at 37°C for 2 hours. After aspirating and washing three times intensively with PBS/Tween-20, 100 μl of 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 the 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 two negative and two positive control serum samples 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 the lung function of patients chronically colonised with P. aeruginosa 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)).

Analysis of data
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. The ability of the serological tests to discriminate between chronic colonisation and no colonisation was therefore estimated using the area under the receiver-operator characteristic curve (ROC area). The difference in discriminatory power between the individual serological tests was estimated by calculating the 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 serological test results.20

The 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). Differences were considered significant if the p value exceeded 0.05. Calculations were performed by Statistical Package for the Social Sciences (SPSS) Version 12.0 (Chicago, IL, USA).

RESULTS
Characteristics of study subjects
The mean (SD) age of the patients in the cross sectional study at the end of the observation period was 14.5 (10.6) years, and for those in the prospective study was 8.1 (2.8) years. Sixty seven patients had chronic colonisation with P. aeruginosa. 60 were intermittently colonised, and 93 patients were not colonised. The characteristics of the study population are summarised in table 1.

The 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 than in non-colonised patients (FEV1 % pred was estimated by calculating the difference in ROC area

Table 1 Characteristics of participants in the cross sectional and prospective studies

<table>
<thead>
<tr>
<th></th>
<th>Cross sectional study</th>
<th>Prospective study</th>
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<tbody>
<tr>
<td>No of participants</td>
<td>220</td>
<td>57</td>
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<tr>
<td>Mean (SD) age (years)</td>
<td>14.5 (10.6)</td>
<td>8.1 (2.8)</td>
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<tr>
<td>Median (range) age (years)</td>
<td>11.8 (0.7–65.4)</td>
<td>7.9 (4.3–14.2)</td>
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<tr>
<td>Male, n (%)</td>
<td>110 (50%)</td>
<td>30 (53%)</td>
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<tr>
<td>P. aeruginosa colonisation status, n (%)</td>
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<tr>
<td>Chronic</td>
<td>67 (31%)</td>
<td>13 (23%)</td>
</tr>
<tr>
<td>Intermittent</td>
<td>60 (27%)</td>
<td>13 (23%)</td>
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<tr>
<td>No colonisation</td>
<td>93 (42%)</td>
<td>31 (54%)</td>
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</table>

Figure 1 Receiver-operator characteristic (ROC) curves of three individual serological tests.
There were no significant differences in discriminatory power between the three 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% v 76%).

Positive serological results for elastase, exotoxin A, and alkaline protease were found in 87%, 79%, and 76%, respectively, of patients with chronic colonisation compared with 42%, 48%, and 17% of patients, respectively, in the intermittently colonised group and 11%, 19%, and 3%, respectively, in the non-colonised group. Combining the results of individual serological tests, 96% of chronically colonised patients had at least one positive antibody titre. Elastase serology was the most sensitive, but six elastase antibody negative patients appeared positive for other P aeruginosa antibodies if a combination of antibody tests was used. The 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%.

### 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.
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P aeruginosa
after acquisition of
previous studies that show worsening of clinical parameters
function, even when adjusted for age. These data confirm
P aeruginosa
colonisation has a significant impact on lung
P aeruginosa
serological test has relatively low sensitivity for the detection
off values according to the manufacturer’s manual, this
colonisation in a large CF population. Using cut
P aeruginosa
To our knowledge, this is the first published study to address
DISCUSSION
population, serological tests against common
P aeruginosa
diagnosis of early
P aeruginosa
seems that the additional value of serological testing for
other signs of
P aeruginosa
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alkaline protease serological tests all discriminate well
between colonisation and non-colonisation. Test character-
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P aeruginosa
Pa, in culture (0 = no, 1 = yes); E, elastase; EA, exotoxin A; AP, alkaline protease (+, positive serological result; –, negative serological result)

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To our knowledge, this is the first published study to address
P aeruginosa
colonisation is a clinically relevant issue;
P aeruginosa
colonisation has a significant impact on lung
function, even when adjusted for age. These data confirm
previous studies that show worsening of clinical parameters
after acquisition of
P aeruginosa.

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.22 23 24 However, 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
characteristics of the serological test. We therefore examined
cultures during three 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 character-
istics depend on cut off values. Instead of using the cut off
values 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 than with the cut off values advised by the
manufacturer. With regard to the clinical relevance of
P aeruginosa
colonisation, the sensitivity of the test needs to be
high. When a positive serological result 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%. Hollings et al25 using a
different ELISA assay, found an elastase sensitivity of only
23% for chronic colonisation in a small study cohort. A
possible explanation for the large difference is the definition
of chronic colonisation used. They used a classification
colonisation status based on cultures over a 6 month period
prior to serological measurement while we defined colonisa-
tion 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, the sensitivity of our test was
much higher. The sensitivity of the other two antibodies is
also higher in our study than in other studies. Exotoxin A had
a sensitivity of 79%, but the specificity was lowest of the three
antigens. Alkaline protease had the lowest sensitivity (76%)
but a very high specificity (97%) and might therefore be
useful for ruling out
P aeruginosa
colonisation.

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

With regard to the use of successive cultures as reference
standard, problems with definition of specificity may arise
because early
P aeruginosa
colonisation might not yet be
proved by positive cultures although the serological test is
positive. On the other hand, a transient positive serological
result without positive cultures was also seen in our follow up
cohort. We did not determine antibody 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.26

In children aged 4–6 years, serological tests were of no
additional value for diagnosing
P aeruginosa
colonisation. Older patients have a higher prevalence of antibodies in the
absence of positive cultures. At ages 6–17 years, only a small
proportion of patients (3–11%) had positive serological
results without a positive culture. The prevalence of
P aeruginosa
colonisation in the youngest patients was lower
than described in recent literature. Burns et al27 showed that
72.3% of patients had evidence of
P aeruginosa
colonisation by
culture and 97.5% by both culture and serology before the age
of 3 years. In the study by Li et al,28 almost 90% of 3 year old
patients had acquired
P aeruginosa
in their cultures.

The prospective data of 57 children show that, in case of
culture conversions to a positive culture, no uniform
serological pattern is seen. From the 13 patients who showed
culture conversion, only five had early positive serological
tests 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 do not seem to be very sensitive. The failure of serological tests to detect early colonisation in young patients emphasises the need for continued reliance on culture. West *et al.* also found that only 54% of children had positive exotoxin A serology before or at the time a culture became positive. In our cross sectional study the antibody that was raised most often in non-colonised patients was exotoxin A, but in the longitudinal follow up it was not clear that exotoxin A titres increased before other titres. The height of the antibody titres could not predict whether early colonisation would become chronic. The cohort may have been too small to show a relation between increasing antibody titres and chronic *P. aeruginosa* colonisation.

In the majority of patients with positive serological tests and negative culture the 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 patients with CF but, except in study settings, they are 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 serological pattern is seen; titres might be raised 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 serological testing with specific antigens.

ACKNOWLEDGEMENTS
The authors thank Hanneke van Deutekom for performing part of the laboratory work.

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Funding: none.
Competing interests: none.

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