

## Detection of antibodies to *Pseudomonas aeruginosa* in serum and oral fluid from patients with cystic fibrosis

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Cystic fibrosis (CF) patients who are chronically infected with *Pseudomonas aeruginosa* make serum antibodies to bacterial surface LPS as well as other pseudomonas antigens. This study investigated the feasibility of using oral fluid samples for the detection of pseudomonas antibodies in CF patients and compared these results with corresponding serum antibodies. Most strains of *P. aeruginosa* produce two forms of LPS molecule, termed A-band (described as a common antigen) and B-band (O-serotype-specific antigen), apparently bound to a common core oligosaccharide moiety. A-band LPS was demonstrated in 45 out of 49 clinical isolates of *P. aeruginosa* by SDS-PAGE and immunoblotting with a specific antibody. Oral fluids were collected from 17 adult CF patients, all of whom were sputum culture positive for *P. aeruginosa* (13 also provided serum samples), 11 primary ciliary dyskinesia (PCD) patients and 37 healthy volunteers. Antibodies to A-band LPS were detected by immunoblotting in all of the CF patients' oral fluids but 10 of the volunteer samples gave weak reactions with immunoblotting. Six of the PCD patients gave a weak reaction with A-band antibodies and only one demonstrated antibodies to core LPS. In a quantitative ELISA, 15 of the 17 CF patients' oral fluids were shown to contain antibodies to A-band LPS, whilst none of the volunteer samples contained antibodies to A-band LPS. All serum samples from the CF patients were positive by both methods. Thus this is a sensitive procedure for the detection of antibodies to A-band LPS of *P. aeruginosa* in oral fluid and serum from patients with CF.

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## INTRODUCTION

*Pseudomonas aeruginosa* causes chronic lung infections in approximately 80% of patients with cystic fibrosis (CF) and this is associated with a dramatic decline in the patients' clinical status (Doring *et al.*, 2000). It is widely agreed that treatment early after initial isolation has been shown both to eradicate the organism and to delay the onset of chronic infection (Frederiksen *et al.*, 1997). Patients who are chronically infected with *P. aeruginosa* produce serum antibodies to the organism, but there is little consensus regarding the clinical value of their measurement, except in those patients where the initial appearance of antibodies predates culture positivity (West *et al.*, 2002). This is due in part to poor standardization of protocols and assays among different centres and some

have relatively poor positive and negative predictive values. Oral fluid samples offer an attractive serological alternative approach to invasive sample collection and are increasingly being used to provide evidence of infections caused by a wide variety of agents (McKie *et al.*, 2002), but not, as yet, for *P. aeruginosa*. The samples are simple to obtain, even in young children, who may not expectorate sputum.

A number of techniques for the detection of serum antibodies to *P. aeruginosa* have been reported utilizing a wide range of antigens including outer-membrane proteins, alginate, enzymes and LPS (Brett *et al.*, 1986; Doring & Høiby, 1983; Fomsgaard *et al.*, 1988; Likavcanova & Lagace, 1992; Rehm *et al.*, 1994). LPS consists of three domains: a hydrophobic, membrane-anchoring lipid A moiety, a conserved oligosaccharide core region and a side chain of repeating oligosaccharide units. In *P. aeruginosa*, two forms of LPS determined by the side chain are recognized: (i) a homogeneous 'A-band' consisting of trisaccharide

Abbreviations: CF, cystic fibrosis; HP, high positive; PCD, primary ciliary dyskinesia.

repeating units of D-rhamnose and (ii) a heterogeneous, high-molecular-mass, serotype-specific 'B-band' (Arsenault *et al.*, 1991; Kocharova *et al.*, 1988; Lam *et al.*, 1989). During pulmonary disease progression, *P. aeruginosa* undergoes phenotypic changes, including changes in LPS expression, with gradual repression of expression of B-band LPS (Hancock *et al.*, 1983). Along with alginate, the major surface antigen of these serotype-deficient strains is A-band LPS, and this is reported to be common amongst strains of the species (Govan & Deretic, 1996; Hatano *et al.*, 1993; Lam *et al.*, 1989).

We report here the detection of antibodies to A-band LPS of *P. aeruginosa* in oral fluids and serum from patients with CF and comparison of the results with those from oral fluids from healthy volunteers and a clinical control group. Approval for patients' samples was given by the Ethics Committee of the Royal Brompton & Harefield NHS Trust/National Heart & Lung Institute, with all subjects giving informed consent.

## METHODS

**Bacterial strains.** Forty-nine isolates of *P. aeruginosa*, each from different CF patients from a number of CF clinics, were investigated for the presence of A-band LPS. Strains of *Burkholderia cepacia* genomovars II, IIIa and IIIb, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Salmonella* Typhi, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Escherichia coli* K-12, *Vibrio cholerae* and *Haemophilus influenzae* were selected from culture collections at the Centre for Infections, HPA, Colindale, London, UK. *P. aeruginosa* strain AK1401, rich in A-band LPS, was a gift from Dr Andrew Kropinski.

**Lipopolysaccharides.** LPS for visualization by SDS-PAGE and silver staining was prepared by digesting whole bacteria with proteinase K (Chart *et al.*, 2003). For immunoblotting, outer-membrane preparations were used (Chart *et al.*, 2002). LPS for coating ELISA plates was purified from outer membranes using hot phenol (Chart *et al.*, 2002).

**Sera.** Pools of sera from CF patients with high pseudomonas anti-cell lysate antibody titres, determined by an in-house whole-cell ELISA, were designated high positive (HP) and comprised sera from a minimum of six patients. Six control serum samples were obtained from healthy non-CF subjects. Rabbit antibodies were raised to LPS prepared from *P. aeruginosa* strain AK1401, under Home Office Licence number 70/5508, using 2 ml Saffan (Schering-Plough) injected intravenously as an anaesthetic. Briefly, a New Zealand White rabbit was challenged with 50 µg purified LPS in Freund's incomplete adjuvant at multiple sites across the back. Three weeks later, the animal was boosted intramuscularly with 50 µg purified LPS in PBS. Seroconversion was confirmed by a test bleed 10 days later and immunoblotting with a panel of *P. aeruginosa* outer-membrane preparations.

**SDS-PAGE and immunoblotting.** SDS-PAGE was performed using an Atto mini-gel apparatus (Genetic Research Instruments). Preparations containing either 83 µg digested cell mass or 10 µg outer-membrane protein were loaded into wells of SDS-polyacrylamide gels (4.5 % stacking gel and 12.5 % separation gel) and electrophoresis was performed (50 mA) for 30 min (Chart *et al.*, 2003). Gels were stained with silver (Tsai & Frasch, 1982) to visualize LPS profiles and photographed, or, alternatively, profiles were transferred to nitrocellulose paper by immunoblotting.

Following electrophoresis, nitrocellulose paper blots were 'blocked' with 3 % skimmed milk in PBS for 5 min at room temperature. Sera (5 µl rabbit serum, 30 µl human serum) and oral fluid samples were added directly to the initial blocking solution and antibodies were allowed to react with the immunoblots for 1.5 h at room temperature before washing in 0.05 % Tween 20/PBS three times for 15 min each. Profiles were reacted with 5 µl per lane of goat anti-human or goat anti-rabbit polyvalent Ig conjugated to alkaline phosphatase for a further 1.5 h at room temperature. The blots were washed three times for 15 min each with 0.05 % Tween 20/PBS before developing with immunoblotting developing solution [6 ml 0.15 M MgCl<sub>2</sub>, 2 ml 0.5 M Tris/HCl (pH 9.5), 2 ml 0.5 M NaCl, 45 µl nitro blue tetrazolium, 35 µl 5-bromo-4-chloro-3-indolyl phosphate].

**Patients.** Seventeen CF patients (four females, group mean age 28.8 years; and 13 males, group mean age 30.4 years) were recruited and all were sputum culture positive for *P. aeruginosa* for at least 6 months at the time of the study. All 17 volunteered oral fluid samples and 13 volunteered sera. Three of the serum samples were taken on the same day as oral fluid was donated, but the mean number of days between collection of the two samples was 114 days ( $\sigma=97$ ,  $n=13$ ), with a maximum of 287 days. Eleven patients with primary ciliary dyskinesia (PCD) (eight females, group mean age 11.3 years; and three males, group mean age 6.1 years) volunteered oral fluid samples. Nine of the patients had never yielded *P. aeruginosa* from sputum and the remainder had not grown this organism for at least 3 years.

**Volunteers.** Samples of control oral fluid were obtained from 37 members of laboratory staff and their families (21 females, group mean age 27.8 years; and 16 males, group mean age 24.1 years). All were considered healthy, with no signs of respiratory infection.

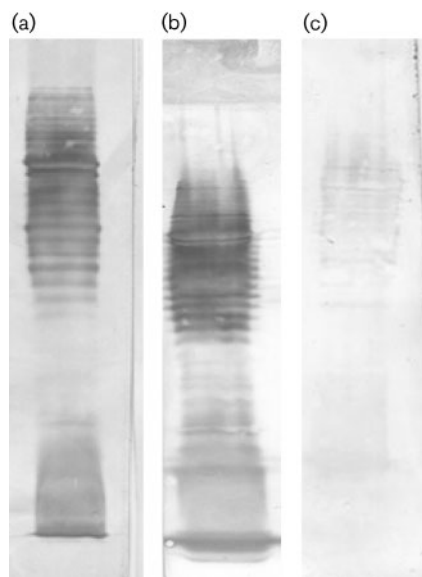
**Oral fluid sampling.** Oral fluid was obtained using Oracol test kits (Malvern Medical Developments). On arrival at the laboratory, the swabs were maintained at room temperature and processed within 3 days. Sponges were detached from the collection swabs, placed in a 5 ml syringe barrel and the fluid was pressed from the sponge. For each sample, 100 µl was set aside for ELISA and the remainder was tested by immunoblot.

**ELISA.** ELISA plates (Greiner Laboratories) were coated with 1 µg LPS per well in 100 µl coating buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 34.5 mM NaHCO<sub>3</sub>, 4 °C overnight) and washed in 0.05 % Tween 20/PBS before blocking with 200 µl 1 % BSA/PBS. Samples were added to LPS-coated wells and to duplicate wells that had been blocked with BSA only. Oral fluids (50 µl per well) or sera diluted 1 : 500 in PBS (100 µl per well) were added and antibody binding was detected with goat anti-human polyvalent Ig conjugated to alkaline phosphatase diluted 1 : 1000 in PBS (100 µl per well). Antibodies conjugated to alkaline phosphatase were detected by adding 200 µl diethanolamine buffer (1 M diethanolamine, 2 mM MgCl<sub>2</sub>, pH 9.6) containing 1 mg *p*-nitrophenol phosphate ml<sup>-1</sup>. The resultant colour was quantified by measuring A<sub>405</sub>. Sample values >0.9 A<sub>405</sub> after 30 min were considered positive (cut-off calculated as mean + 3 ×  $\sigma$  of volunteer oral fluid samples and human negative control sera).

**Statistical analyses.** Student's *t*-tests were performed to compare the ELISA results from the CF and control populations for oral fluid and serum samples.

## RESULTS AND DISCUSSION

SDS-PAGE and silver staining of whole-cell digests of the O-serotype strains demonstrated long-chain LPS in most of the strains. To visualize A-band LPS, profiles were



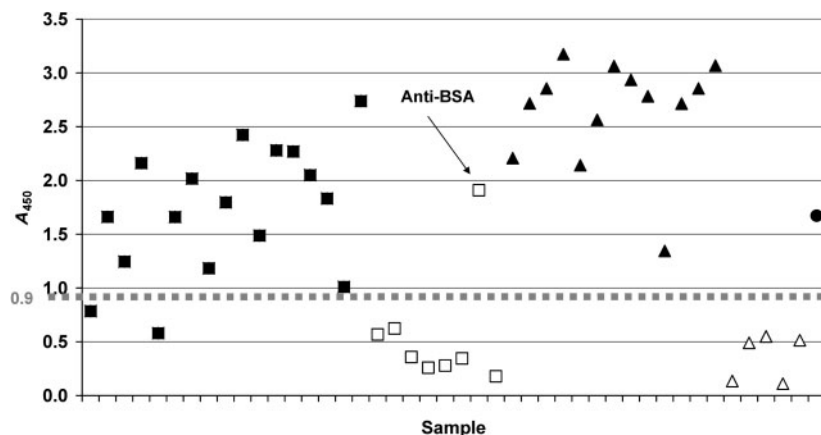
**Fig. 1.** Antibody-antigen reactions with outer-membrane preparations of *P. aeruginosa* strain AK1401 using CF patient sera (a), CF patient oral fluid (b) and volunteer oral fluid (c).

immunoblotted with the hyperimmune rabbit antiserum to strain AK1401, which expresses A-band LPS. Fifteen of the 20 O-serotype strains (O1–O6, O9–O11, O13 and O16–O20) were found to express A-band LPS, as were 45 out of 49 clinical strains. These results confirmed that the majority of *P. aeruginosa* clinical strains produce A-band LPS and have the potential to elicit an A-band-specific antibody response in animals (Lam *et al.*, 1989). Interestingly, the type strains listed above also reacted with the control HP serum pool, suggesting that the main serum LPS antibody response in patients with CF was to the A-band. None of the O-serotype strains reacted with the healthy human control sera; similarly, the pooled HP CF sera failed to react with the other bacterial species tested.

Each of the 13 CF patient serum samples was found to contain antibodies to A-band and core LPS by immunoblotting (Fig. 1a) and ELISA (Fig. 2) and all 17 CF oral fluid samples were shown to contain antibodies to A-band LPS by immunoblotting (Fig. 1b). Fifteen of the 17 oral fluids also contained antibodies to A-band LPS by ELISA, giving the ELISA a sensitivity of 1.00 with serum samples and 0.88 with oral fluids. Oral fluid samples from the PCD patients were only tested by immunoblotting with A-band LPS and six of them gave weak antibody reactions with A-band. Moreover, one of these subjects expressed antibody to the core LPS of AK1401, despite not having been culture positive for the past 3 years.

Ten of the 37 healthy volunteers had low levels of oral fluid antibodies binding to A-band LPS, as illustrated by faint bands in immunoblots (Fig. 1c), and one of the volunteers (a *Pseudomonas*-laboratory worker) had oral fluid antibodies to A-band LPS, as indicated by a strong immunoblot reaction, which included antibodies to core LPS. Oral fluids from eight healthy volunteers were tested by ELISA (Fig. 2) and seven of these samples (including two that had demonstrated faint bands by immunoblot) were shown not to contain antibodies to A-band LPS. The outlier was revealed to be due to non-specific antibodies binding to BSA, as this sample was immunoblot negative for A-band LPS. Statistical analyses comparing the ELISA results from the CF patients and the healthy volunteers showed that there was a significant difference between the two populations for both the serum ( $P=0.0001$ ) and oral fluid ( $P=0.0001$ ) samples.

The observation that antibodies from oral fluid may bind non-specifically to BSA illustrates the importance of ensuring that immunoassays include a BSA control to avoid false-positive results. The salivary carriage rate of *P. aeruginosa* in hospital patients and normal controls has been reported as approximately 5% (Botzenhart *et al.*, 1985), but it is not known if such colonization leads to stimulation of circulating antibodies. Antibodies to several O-serotype LPS antigens have been detected in human sera



**Fig. 2.** Summary of A-band LPS antibody levels in CF patient and healthy volunteer samples as determined by ELISA. ■, CF oral fluid; □, healthy volunteer oral fluid; ▲, CF sera; △, healthy volunteer sera; ●, pooled HP CF sera.

(Grzybowski *et al.*, 1989), but the levels in the general population, specifically to A-band LPS, remain unknown. As *P. aeruginosa* is ubiquitous, it seems likely that healthy subjects are exposed to environmental strains and mount an antibody response.

Antibodies to the core LPS moiety of strain AK1401 were demonstrated in all 17 CF patients, one PCD patient (who had grown *P. aeruginosa* from sputum 3 years previously) and one of the volunteers, who had had occupational exposure to *P. aeruginosa* in the laboratory for several years. In our experience, the appearance of anti-core LPS antibodies most often suggests prolonged infection (unpublished results) and explains, in part, the immunoblot reactions presented here.

It was observed during the course of this study that the oral fluids collected from patients with CF were of a significantly lower volume than samples collected from either control group. This may suggest that the oral fluids of CF patients are more concentrated or that they are as concentrated as controls but contain fewer antibodies. We are currently developing a quantitative assay to explore this observation.

The immunoblotting data presented were obtained with a polyvalent anti-human antibody. Further blots (two per Ig class) were performed employing paired oral fluid and sera to investigate the class of the A-band LPS-specific antibodies collected from CF patients. Strong banding patterns were observed with both anti-human IgA and IgG secondary antibodies and weaker bands were seen when blotted with an anti-human IgM antibody for both oral fluids and sera.

The fact that the oral fluid results corresponded closely to the serum results was promising. Current studies involve screening a much larger cohort of healthy volunteers in order to calculate an ELISA cut-off value with more precision, thus hopefully reducing the number of false-negative ELISA results such as the two CF oral fluid outliers in Fig. 2. However, immunoblotting oral fluids against AK1401 LPS differentiated positive and negative samples, with all CF patients demonstrating a strong antibody response to A-band and core LPS. Although the presence of antibodies was not necessarily indicative of a current infection, the detection of initial responses and subsequent rises in levels is of potential clinical value and oral fluids may provide a convenient means for the frequent monitoring of the antibody response to *P. aeruginosa* in CF patients. Further prospective longitudinal studies are needed to address the clinical utility of this approach.

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