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Oropharyngeal production of pneumococcal capsular antigen and the potential for contamination of expectorated sputum samples in pneumococcal pneumonia

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SUMMARY

The presence of pneumococcal capsular antigen (PCA) in the oropharynx was sought in subjects without respiratory tract infection. Saliva specimens from 239 subjects were analysed by counter-current immunoelectrophoresis using 'Omni-serum'. 15·5% gave positive reactions but only 24% of positive samples were typable and therefore due to pneumococcal or pneumococcal-like antigens. Given that oropharyngeal production of antigens occurs we investigated whether PCA in expectorated sputum arose from oropharyngeal contamination. Sixteen patients with pneumococcal pneumonia, and with sputum positive for PCA, were investigated in detail. On the basis of serotyping and concentration the PCA in sputum was thought to arise from the lower respiratory tract in all cases. This was confirmed by a simple, novel approach involving the comparison of concentrations in concomitant samples of saliva and sputum. Thus while oropharyngeal production of antigens poses a potential diagnostic problem the latter approach can be used to exclude contamination.

INTRODUCTION

The pathogens responsible for community-acquired pneumonia are often not identified; in most hospital series identification has been achieved in only 33–50% of cases [1]. The pathogen most likely to be missed is *Streptococcus pneumoniae* as it is the commonest causative agent of community-acquired pneumonia. With detailed laboratory investigations it can be found in 76% of cases admitted to hospital [2] but in routine clinical practice it is found less frequently. Increasing the diagnostic sensitivity of methods for detecting *S. pneumoniae* would contribute significantly to the overall diagnostic rate for pneumonia and permit appropriate antibiotic treatment.

Streptococcus pneumoniae elaborates a repertoire of immunogenic toxins and surface markers. Recognition of these can provide evidence of infection. The

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immunological response to the toxin, pneumolysin, has both a high sensitivity and specificity in diagnosis but because assays depend on the appearance of antibodies. results are delayed [3, 4]. More immediate recognition is obtained with immunoassays for pneumococcal antigens; those which have received most attention being the C-polysaccharide (PnC) and the capsular polysaccharide antigens (PCA). These antigens have been detected in sputum, pleural fluid. serum, urine and cerebrospinal fluid [5–7]. Antigen-based assays are rapid. permitting results on the same day and, when positive, provide a diagnosis in culture-negative cases [8].

There are at least three settings in which an antigen-based diagnosis of *S. pneumoniae* would be beneficial. Firstly rapid results which can direct initial antibiotic therapy will be useful for patients admitted to hospital with severe pneumonia and who run the risk of mortality rates of 6–30% [9, 10]. Secondly in performing studies, specific diagnosis will help in accruing sufficient numbers of documented pneumococcal pneumonias to achieve statistical significance, which has proved difficult in the past for example in relation to pneumococcal vaccine studies [11]. Thirdly in the tropics, where respiratory tract infection, often pneumococcal, is now the principal cause of mortality in children under 5 years old [4, 12] a simple and rapid test for *S. pneumoniae* in a dispensary or hospital might help in the management of an unwell, febrile child.

The presence of pneumococcal antigens in pleural fluid, serum and urine is taken as a specific indication of pneumococcal infection but these samples do not generally prove sufficiently sensitive; urine is positive in up to two-thirds of cases [2] and serum is positive in less than half [13]. Pneumococcal antigen is most frequently found in sputum, 68–86% of cases [7, 9], but the specificity is in doubt. As *S. pneumoniae* can reside in the oropharynx the presence of pneumococcal antigen in sputum may result from oropharyngeal contamination rather than a lower respiratory tract pathogen. The prevalence of oropharyngeal production of pneumococcal antigens is not known. We have investigated the occurrence of PCA in the oropharynx and the likely source of PCA in sputum from microbiologically documented cases of pneumococcal pneumonia, describing ways to distinguish contamination from true positive results.

MATERIALS AND METHODS

Patients with community-acquired pneumonia

For the purpose of the study pneumonia was defined as an acute lower respiratory tract infection with fresh, previously unrecorded shadowing on a chest radiograph. Adult patients with pneumonia, identified clinically on admission, or as a result of laboratory isolation of *S. pneumoniae* from blood culture were visited as soon as possible after admission and their clinical details recorded. Samples of saliva, sputum and urine were collected daily while serum was collected on alternate days throughout their hospital stay. When possible, samples were also collected at the first outpatient follow-up. Saliva was obtained prior to expectoration of sputum. Samples of saliva and sputum were collected in the presence of the investigators (PV, CT) and, when necessary, with assistance from a physiotherapist. Salivary samples containing purulent material were not

accepted. Only purulent or mucopurulent samples of sputum were accepted for analysis. Samples were stored immediately at −20 °C for later analysis.

Investigations

Specimens of sputum sent for culture were microscopically assessed to confirm their quality. Blood and sputum were cultured and serum tested by immunofluorescence for antibodies to *Legionella pneumophila* serogroup 1 and for complement-fixing antibodies to influenza virus A and B, respiratory syncytial virus, adenovirus, *Coxiella burnetii*, *Chlamydia psittaci* and *Mycoplasma pneumoniae*. Antibodies to *Chlamydia pneumoniae* were not sought. Chest radiographs were taken on admission, at about 7 days and at the first outpatient follow-up.

Subjects without pneumonia

Saliva, and when possible sputum, were collected from healthy volunteers and from hospital inpatients and outpatients, with and without respiratory disease. Clinical details, smoking history and medication, including inhaler usage and recent antibiotic treatment, were recorded.

Counter-current immunoelectrophoresis (CIE)

Samples were analysed for PCA by CIE based on a method described by El Refaie and Dulake [14]. Sputum was homogenized, volume for volume, with 2% *N*-acetylcysteine. Urine was concentrated overnight with polyacrylamide absorbent gel, PAG (Sigma, Poole, UK). Ten millilitres of molten agar in veronal-acetate buffer, pH 6·6, was plated onto 10 × 8 cm grease-free glass plates. Rows of wells were cut in the agarose gel with each well being 3 mm diameter and the rows 5 mm apart. Two microlitres of each sample and two microlitres of 'Omni-serum' (Statens Serum Institut, Copenhagen) were placed in wells on opposing rows. Electrophoresis at 200 V was carried out for 20 min using a barbital buffer, pH 8·6, with the 'Omni-serum' at the cathodal end. At the end of electrophoresis the gel was examined for a precipitin band. To check for the further development of precipitation a reading was also taken after overnight incubation at 4 °C. All negative samples were diluted 1:10 to check for a prozone effect.

Serotyping

Serotyping of PCA positive samples was carried out by CIE using monovalent antisera (Statens Seruminstitut, Copenhagen, Denmark) to types 1, 3, 4, 6, 8, 9, 19, 22 and 23. This range covered 73% of blood culture isolates seen in our department over the preceding 2 years. Blood culture isolates were kindly serotyped by the Pneumococcal Reference Laboratory, PHLS, Colindale, London.

One batch of monovalent sera was used throughout the study. However, due to a higher turnover, several batches of 'Omi-serum' were used which meant that for some samples the latter may have been fresher with a higher sensitivity than the monovalent sera for PCA.

Titrations

The concentration of PCA was calculated from end-point titres with the relevant monovalent antiserum. The limits of detection for the monovalent antisera were determined by titration with 'Pneumovax' (Thomas Morson,

Hoddesdon, UK), which contains 50 mg/l of each of 23 serotypes of pneumococcal capsular polysaccharide. Titrations were not carried out on concentrated urine because the concentrating effect of PAG was uncontrolled and variable.

Absorption of 'Omni-serum'

Streptococcal isolates from commensal oropharyngeal flora were cultured in brain heart infusion broth (Oxoid, UK). Broth cultures were centrifuged and the bacterial sediment resuspended in 'Omni-serum' (v/v) for 48 h at 4 °C. Absorbed 'Omni-serum' was retrieved by centrifugation and collection of the supernatent. Successful absorption was confirmed by the elimination of the reaction with the isolate.

RESULTS

Patients with pneumonia

Sixteen patients were studied. On the basis of British Thoracic Society criteria [7] 10 patients had 'definite' pneumococcal pneumonia with pneumococcal bacteraemia (8 patients) and/or antigenaemia (9 patients). Six patients had 'probable' pneumococcal pneumonia with PCA in sputum. The age range was 28–78 years (median 60 years) and there were 12 males. The duration of symptoms prior to admission ranged from 3 to 35 days (median 7 days). All patients had respiratory symptoms (cough, 15; dyspnoea, 15; expectoration, 16; pleuritic pain. 11; fever, 9). Eight patients had a past history of chronic respiratory disease; 6 patients had chronic bronchitis, 2 had asthma and single patients had Milroy's syndrome and inactive pulmonary tuberculosis. On admission, 11 patients were febrile with a temperature > 37 °C and all patients had clinical signs of consolidation.

Investigations

Streptococcus pneumoniae was isolated from 3 sputum cultures (patients 5. 6 and 13) and 8 blood cultures. Cultures and serological testing did not reveal any other pathogens. Culture of empyema fluid in one case (patient 14) was negative.

Radiology

Consolidation was present in 1 lobe in 13, 2 lobes in 1 and 3 lobes in 2 patients. Pleural effusions developed in 4 patients and 1 of these developed an empyema. In 9 patients some radiological improvement had occurred by about 1 week.

Distribution and serotypes of PCA

One hundred specimens of saliva, 72 sputa, 73 sera and 105 urines were collected from patients with pneumonia. The distribution of PCA in these body fluids and their serotypes are shown in Table 1. The table shows that, when identified. there was concordance of serotypes between samples and also with blood culture isolates. Pleural fluid from one patient (patient 9) contained PCA. The failure of monovalent sera to detect PCA in a few 'Omni-serum' positive samples may reflect the relative sensitivity of these antisera.

Table 1. *The presence of pneumococcal capsular antigen and its serotype in various body fluids in 16 patients with pneumococcal pneumonia*

Pt no	Serotype	Blood culture	The presence (+) or absence (−) of PCA in Saliva	Sputum	Serum	Urine
1	3	—	+ (3)*	+ (3)	—	—
2	3	—	—	+ (3)	—	—
3	4	—	+ (4)	+ (4)	—	—
4	1	—	+ (ins)	+ (1)	—	—
5	3	+ (3)	+ (−)	+ (3)	+ (3)	+ (3)
6	3	—	+ (3)	+ (3)	+ (3)	+ (3)
7	9	+ (9)	+ (−)	+ (9)	+ (9)	+ (−)
8	1	—	+ (ins)	+ (1)	—	—
9	19	+ (19)	+ (19)	+ (19)	+ (19)	+ (19)
10	?	—	—	+ (ins)	—	—
11	4	+ (4)	+ (ins)	+ (4)	+ (4)	+ (4)
12	1	+ (1)	+ (1)	+ (1)	+ (−)	+ (−)
13	3	+ (3)	—	+ (3)	+ (3)	+ (3)
14	?	—	—	+ (?)	+ (?)	+ (?)
15	4	+ (4)	NS†	+ (−)	+ (4)	+ (4)
16	1	+ (1)	+ (−)	+ (1)	—	+ (1)
Total positives		8	11	16	9	10

(*) serotype; (?) serotype not identified; (ins) insufficient sample for further testing; (−) no reaction with monovalent antiserum. † (NS) no specimen.

Kinetics of PCA

The limits of detection of five monovalent antisera, types 1, 3, 4, 9 and 19, used for titration were 0·6, 0·3, 0·6, 0·2 and 0·1 mg/l respectively. Representative graphs from two patients are shown in Fig. 1(*a*, *b*). Concentrations as high as 360 mg/l were found. PCA disappeared from sputum and saliva after a median duration of 5 days and mainly within 10 days. In three patients there was a late appearance and/or a rise in the concentration of serum PCA, even after effective treatment and clinical improvement. PCA disappeared from serum and saliva after a median duration of 10 days. At outpatient follow-up four patients still had positive specimens. These patients were aged 51, 61, 68 and 74 years and clinically had moderate to severe episodes of pneumonia requiring hospital stays of 9, 11, 20 and 21 days. One of these four patients still had positive saliva (on day 29 post admission), two had positive sputum (days 29 and 40) and three had both positive serum and urine (days 29, 40 and 56). The serotypes concerned were type 3 in three cases and type 9 in one case.

There were ten paired samples of positive sputum and positive saliva. The concentration of PCA in sputum exceeded that in saliva in all cases. The median ratio of PCA concentrations in sputum to saliva was 16:1 (range 3·2:1 to 1000:1). There were 20 paired samples in which sputum was PCA positive but saliva was negative. In only three paired samples was sputum PCA negative while saliva was positive. The latter pairs occurred at a time when pneumonia was resolving and sputum expectoration was coming to an end. The concentrations of PCA in saliva in these three cases was very low at 0·1 mg/l and on typing all three salivary samples contained type 19 antigen.

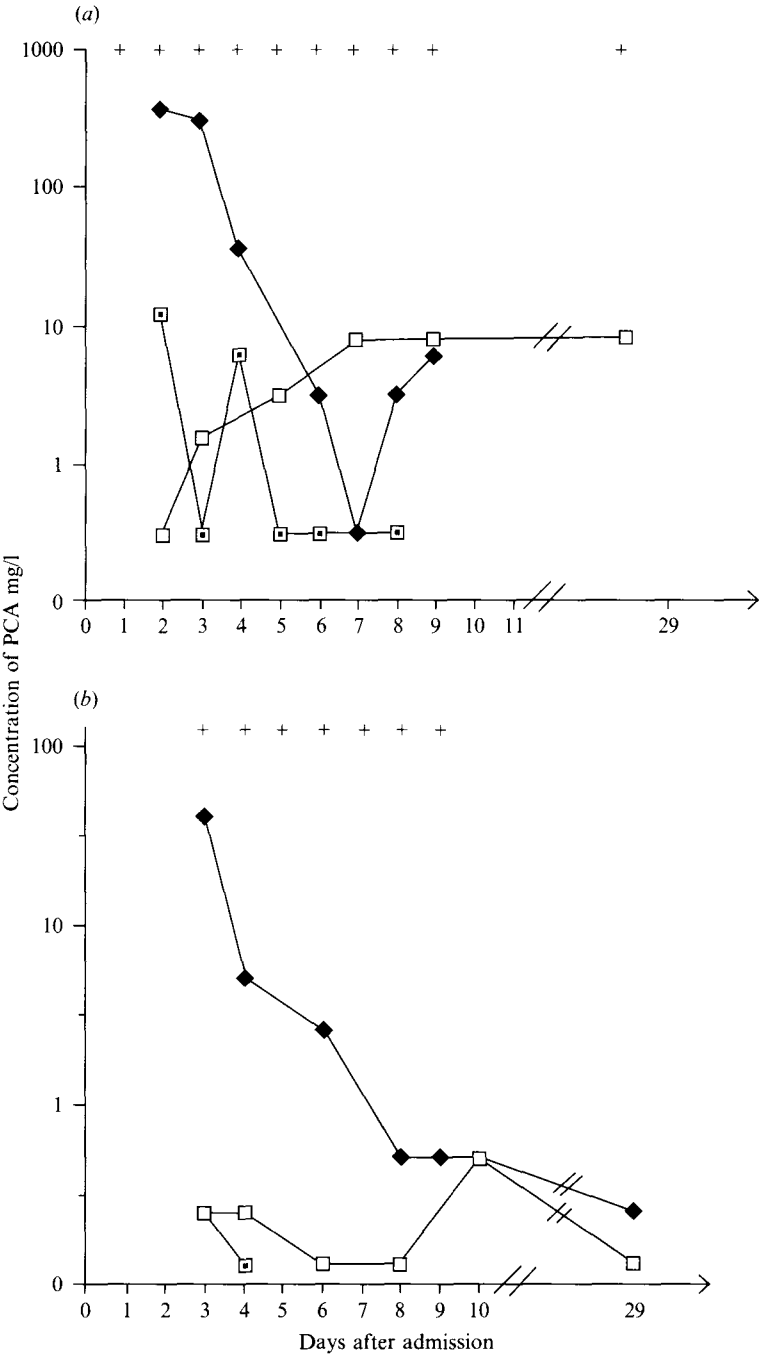

Fig. 1. Changes in the concentration of pneumococcal capsular antigen (PCA) with time in various body fluids following the admission of two representative patients. (*a*) Patient 6; (*b*) patient 7. The presence of PCA in urine is indicated by a + sign. ⊡, Saliva; ◆, sputum; ■, serum.

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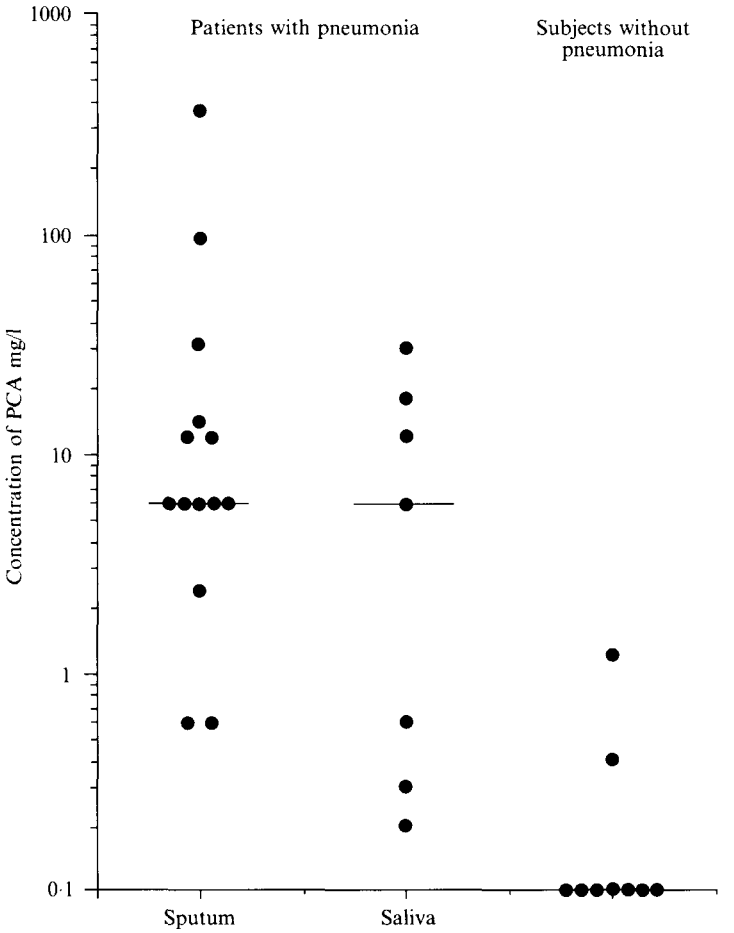

Fig. 2. The concentration of pneumococcal capsular antigen (PCA) in initial positive specimens from patients with pneumonia and in saliva from subjects without pneumonia. Bars indicate median values.

Subjects without pneumonia

Thirty-one healthy volunteers (median age 31 years, range 20–72), 115 patients without respiratory disease (median 58 years, range 19–81) and 93 patients with respiratory disease (median 62 years, range 16–90) were recruited. The most common respiratory diseases were chronic bronchitis (37) and asthma (27). Twenty-four patients had received an antibiotic in the preceding week. All subjects provided specimens of saliva and 32 patients provided sputum.

Specimens gave positive reactions with 'Omni-serum' in a total of 37 (15·5%) cases: 21 (18·3%) of those without respiratory disease and 16 (17·2%) of those with respiratory disease. Only one specimen of sputum was positive. In 27 cases these reactions were eliminated or reduced following absorption of 'Omni-serum' with 'viridans streptococci'. Only nine specimens (3·8% of all saliva specimens) were typable with the monovalent sera available, all proving to be type 19. In Fig.

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2 the concentrations of antigen in these nine are compared with the concentrations in initial specimens from patients with pneumonia.

There was no relationship between positivity and smoking history, inhaled steroids or recent antibiotics.

DISCUSSION

Streptococcus pneumoniae is a recognised resident in the upper respiratory tract [15], being found in the oropharynx of 35% of pre-school children, 20% of healthy adults and up to 40% of patients with chronic bronchitis [16, 17]. Hendley and co-workers found that the most common serotype was type 19 [16]. The release, by these oropharyngeal organisms, of antigens into saliva has not been studied extensively. Krook and colleagues assayed saliva from 25 surgical patients and were unable to detect PnC [18]. In contrast our study of a large number of subjects, without respiratory tract infection, has shown that 15·5% of saliva samples give a positive reaction with 'Omni-serum'. However some of these reactions may be attributed to cross-reactions with non-pneumococcal antigens. Such cross-reactions are well documented with other oral microorganisms [19, 20] and we have demonstrated that prior absorption of 'Omni-serum' with 'viridans streptococci' eliminates some (73%), but not all, of these positive reactions. About 4% of all saliva samples contained antigen which we were able to type, proving to be type 19 in all cases. When antigen is typable it is very likely to be pneumococcal in origin but one cannot exclude particular cross-reactions between type 19 pneumococci and other organisms. Nevertheless we have demonstrated that pneumococcal or pneumococcal-like antigen is detectable in saliva from a significant proportion of subjects.

The presence of antigens in the oropharynx poses a diagnostic problem. Whenever antigen is found in sputum one cannot be certain whether it comes from the lower respiratory tract or from oropharyngeal contamination. Therefore, in using the presence of antigen in sputum for the diagnosis of a lower respiratory tract pneumococcal infection, it is difficult to distinguish between a true positive and a false positive result. A further problem in defining false positive reactions is that, on detailed investigation, about 9% of pneumonias are dual infections involving *S. pneumoniae* and another pathogen [9]. Therefore when there is clear evidence of a non-pneumococcal pathogen one cannot be certain whether pneumococcal antigen in sputum represents a false positive, from oropharyngeal contamination, or a true positive, from a dual infection. These problems hamper the calculation of the specificity of sputum pneumococcal antigen for the diagnosis of a lower respiratory tract infection. Before the specificity can be calculated the true source of pneumococcal antigen in sputum must be identified. We have attempted this for PCA in our patients with pneumonia.

In the past serotyping has been used to determine the significance of sputum PCA. If PCA in sputum comes from the lower respiratory tract it should be of the same serotype as PCA in serum and urine, and the same as a blood culture isolate. We found this to be the case in all eight patients in whom a complete set of positive results was available. This is in agreement with the findings of Tugwell and Greenwood [5]. They found a concordance of serotypes between sputum, serum

and urine in 28 out of 29 patients with positive samples. However serotyping cannot be used to assess positive sputum in non-antigenaemic and non-bacteraemic patients. This group represents a significant proportion of patients with suspected pneumococcal pneumonia.

That sputum antigen can be serotyped is reasonable evidence that it is PCA, as opposed to a cross-reacting antigen. Oropharyngeal contamination is then less likely, but still possible given that we found PCA production in the oropharynx. Thus serotyping alone does not provide conclusive evidence of the significance and source of sputum antigen.

A quantitative cut-off level has been suggested to distinguish between contamination and true positive samples [21]. The concentration of pneumococci in pneumonic lung must be considerably greater than that in the oropharynx. Therefore one expects the concentration of antigen in the lung to be much greater than in saliva. Contaminated sputum samples would then have only low concentrations of antigen. Parkinson and colleagues have used 0·5 mg/l as a cut-off concentration for PnC and found a good association with sputum culture of *S. pneumoniae* [21]. We have found that the concentration of PCA in sputum from patients with pneumonia is higher than in saliva from subjects without respiratory infection. The latter group of subjects was well at the time of sampling and it may be that oropharyngeal production of 'Omni-serum' positive antigen increases during pneumonia, with a change in oral hygiene and the well-being of the patient. However it is uncertain whether the levels we have found in sputum would be reached in saliva.

On the basis of serotyping and absolute concentration it would seem that sputum PCA in our patients comes from the lower respiratory tract, but we have used a novel approach to confirm this. This approach has been to take paired samples of saliva and sputum from our patients and compare the concentration of PCA. We have compared concentrations in absolute terms but the comparison can also be made in terms of end-point titres with 'Omni-serum'. This obviates the need for serotyping. If PCA negative, expectorated sputum is contaminated by PCA positive saliva one expects sputum to dilute the PCA concentrations in the saliva. The concentration of sputum PCA would then be less than in saliva. In 30 paired samples we found the reverse, implying that sputum contained PCA in excess of the amount in saliva. This can only be explained on the basis of sputum containing PCA before passing through the oropharynx and therefore PCA being derived from the lower respiratory tract. Thus the differential comparison of PCA in saliva and sputum is a logical way of determining the source of PCA. It can be performed in patients who are not bacteraemic or antigenaemic and does not require serotyping or the measurement of absolute concentrations. Consequently it is a relatively simple and widely applicable way of determining the significance of sputum PCA.

The presence of PCA in saliva during pneumonia has not been described before. Krook and co-workers found PnC in 55% patients with pneumococcal pneumonia, diagnosed on the basis of a positive sputum or blood culture [18]. In this study we have found PCA in saliva in 68% of our patients. These pneumococcal antigens could arise from local oropharyngeal production, contamination by expectorated sputum or be transported from pneumonic lung. Such transport could be achieved

via the mucociliary escalator or through blood and excretion through the salivary glands. It is not possible from this study to reach a conclusion on the origins of salivary PCA during pneumonia. Further work on this is warranted as 30% of patients with pneumonia have an unproductive cough on admission to hospital [9]. If salivary PCA can indicate a lower respiratory tract pneumococcal infection saliva could provide useful information in those patients who do not provide a sputum sample.

In the course of this study we have confirmed two findings made previously. Firstly PCA can persist for a considerable duration in body fluids, and be detectable long after the clinical resolution of pneumonia. Van der Auwera and colleagues were able to detect PCA in the urine of two patients for 25 weeks following hospital admission [6]. Our follow up only extended to the first outpatient appointment, 4–6 weeks following admission, and a few patients still had PCA positive samples. In general PCA was cleared from saliva and sputum before serum and urine. Secondly we have found that there can be a late rise in the concentration of PCA in serum. This has been described in an animal model, in which PCA was given intravenously to mice [22]. The late rise was attributed to the recirculation of antigen from tissue depots but in our clinical cases the kinetics of release of PCA from pneumonic lung also has to be considered.

In conclusion we have demonstrated the presence of pneumococcal or pneumococcal like antigens in saliva, in the absence of active respiratory infection. This raises the potential problem of contamination of expectorated sputum samples. However in our patients with pneumonia we have excluded contamination of positive sputum specimens on the basis of serotyping, concentration and the comparison of concomitant saliva and sputum. The latter approach has potential as a simple and widely applicable method for determining the significance of sputum PCA. As such it could be used in larger studies to calculate the specificity of sputum PCA in the diagnosis of pneumococcal lower respiratory tract infection.

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