Detection of pneumolysin in sputum

JANICE WHEELER*†, ROGER FREEMAN*, MICHAEL STEWARD†, KIRSTINE HENDERSON†, MAUREEN J. S. LEE†, NIGEL H. PIGGOTT†, GARY J. A. ELTRINGHAM* and ANGELA GALLOWAY*

*Newcastle Regional Public Health Laboratory, Westgate Road, Newcastle upon Tyne NE4 6BE and †Novocastra Laboratories Ltd, Balliol Business Park West, Benton Lane, Newcastle upon Tyne NE12 8EW

Western blot detection of the species-specific pneumococcal product, pneumolysin (SPN), was shown to be almost as sensitive as PCR for the non-cultural detection of pneumococci in 27 Streptococcus pneumoniae culture-positive sputa from patients stated to have chest infections. Both techniques were considerably more sensitive than counter-current immuno-electrophoresis for pneumococcal capsular polysaccharide antigens (CPS-CIE) on the same specimens. Sensitivities for PCR, SPN-immunoblotting and CPS-CIE were 100%, 85% and 67%, respectively. In 11 S. pneumoniae culture-negative sputa taken from patients receiving antibiotics, but with proven recent pneumococcal infection, PCR and SPN-blot were positive in six (in two of which CPS-CIE was also positive), PCR alone was positive in one and SPN-blot alone was positive in one. In 11 S. pneumoniae culture-negative samples from patients not receiving antibiotics and with no known recent pneumococcal infections, one or more non-cultural test was positive in 11. Although further evaluation is required to assess the significance of pneumolysin detection in relation to carriage and infection and to devise a more suitable test format, these preliminary studies suggest that pneumolysin detection is a promising new approach to the non-cultural diagnosis of pneumococcal chest infection.

Introduction

Streptococcus pneumoniae (the pneumococcus) is the commonest aetiological agent of community-acquired pneumonia in all age groups, accounting for up to 50% of hospital admissions of cases in which an aetiology is proven [1]. The majority of the 33% of cases in which no agent is identified are also probably of pneumococcal origin [2]. Culture of S. pneumoniae (from respiratory secretions or from blood) confirms the diagnosis, but culture is often compromised by prior antibiotic therapy. Therefore, non-cultural methods for the diagnosis of pneumococcal chest infection are desirable, but currently available techniques have a number of limitations.

Detection of the type-specific pneumococcal capsular polysaccharide (CPS) antigens in respiratory secretions, urine and serum by either counter-current immuno-electrophoresis (CPS-CIE) or particle agglutination techniques is widely used, utilising ‘Omniserum’ (Statens Seruminstitut, Copenhagen, Denmark), a mixture of rabbit polyclonal antibodies to 83 capsular serotypes—although Omniserum is known to cross-react with antigens of viridans (non-pneumococcal) streptococci [3]. In urine and serum these tests have high specificity but poor sensitivity, whereas in respiratory secretions problems arise with both [4]. PCR on sputum has been described [5], but the cost of PCR-based methods precludes their use in a routine clinical laboratory setting at present.

Pneumolysin (SPN) is a species-specific 53-kDa protein found in all strains of S. pneumoniae and is an ideal target for antigen detection. There is good experimental and clinical evidence that SPN is present in large amounts in pneumococcus-induced inflammatory processes, in which it may be an important virulence factor [6]. The protein nature of SPN permits the development of recombinant antigens and the production of monoclonal antibodies (MAbs) for use in its detection. Therefore, this study examined the feasibility
of using immunodetection of pneumolysin as the basis of a non-cultural test for the presence of pneumococci.

Materials and methods

Patients and organisms
Detection of SPN by Western blot analysis (SPN-blot) was attempted in 65 purulent or mucopurulent sputa from patients stated to have clinically significant respiratory infection. Twenty-seven of these samples were culture-positive for *S. pneumoniae*, 18 yielding a pure, heavy culture. *Haemophilus influenzae* was co-isolated from four samples and other organisms (*Candida* spp. in two instances, *Proteus mirabilis* in two and *Staphylococcus aureus* in one) were co-isolated in the remaining five samples, although *S. pneumoniae* was the predominant organism. None of these patients was receiving antibiotics. The other 38 samples were culture-negative for *S. pneumoniae*, despite the use of a highly selective medium [7], although other organisms were isolated from some, such as coliforms (eight samples), *H. influenzae* (seven), *Staph. aureus* (four), *Candida* spp. (two) and mixed respiratory tract commensals (three). Twenty-seven of these latter patients were receiving antibiotics at the time of sampling, and *S. pneumoniae* had been isolated from 11 of these within the previous 2–10 days. All specimens were routinely digested with dithiothreitol (‘Sputasol’; Oxoid, Basingstoke) before culture, CPS-CIE, SPN-blot and PCR targeting the pneumolysin gene (SPN-PCR).

**CPS-CIE**

This was performed by a standard method [8] with Omniserum.

**SPN-blot**

Specimens were centrifuged (1500 *g* for 5 min) and the supernates were diluted, if necessary, with distilled water to a protein concentration of c. 2 mg/ml. Each was then mixed with an equal volume of reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, containing SDS 2%, glycerol, 20%, mercaptoethanol 5% and bromophenol blue) and boiled for 4 min; 10-μl volumes of each treated sample were then run on SDS-PAGE 8 or 10% gels, the separated proteins were then transferred to nitrocellulose by standard techniques. Blots were blocked by incubation with non-fat dried milk 10% in rinse buffer (10 mM phosphate-buffered saline containing Tween 20 0.05%) and successively incubated for 1 h at room temperature with a murine MAb to recombinant SPN (NCL-SPN 9.1/2/36; Novocastra Laboratories, Newcastle upon Tyne) and alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins (Dako, Cambridge). Both antibodies were diluted in rinse buffer containing fetal calf serum 10%. The reaction was developed with nitroblue tetrazolium/bromochloroindolyl phosphate (NBT/BCIP) in alkaline phosphatase buffer (100 mM NaCl, 100 mM diethanolamine, 5 mM MgCl₂, pH 9.5) for up to 10 min at room temperature. Blots were washed three times in rinse buffer between each stage of the procedure. SPN was detected as a band at c. 53 kDa. The MAb used has been shown not to react with C-reactive protein (CRP) or streptolysin O (SLO) (data not shown).

**SPN-PCR**

DNA, extracted by phenol-chloroform treatment and purified on diatomaceous earth, was taken up into PCR-grade water and used as the template in the PCR. The PCR used an *S. pneumoniae* primer set (NCL-SPPS; Novocastra Laboratories) which comprises a pair of oligonucleotide primers to amplify a region of 518-bp from the pneumolysin gene and an internal (amplification) control which co-amplifies with the same primers to yield a product of 300 bp, thus aiding in the detection of false-negative results. The manufacturer’s instructions were followed. Amplified products were detected by running 5-μl volumes on an agarose 3% gel (‘Aquapor’; National Diagnostics, Atlanta, GA, USA), staining with ethidium bromide, examining with fluorescence and photographing under UV light. The SPN-PCR does not amplify with other species of α-haemolytic streptococci, staphylococci or a range of other common bacterial species.

**Results**

The results obtained with all three tests for the 27 *S. pneumoniae* culture-positive and 38 culture-negative sputa are summarised in Table 1. Representative results for the SPN-blot and PCR are shown in Figs. 1 and 2, respectively. Of the 27 *S. pneumoniae* culture-positive sputa, 18 were positive by all three tests, a further five were positive by PCR and SPN-blot but not by CPS-CIE and another four were positive by PCR alone, giving a sensitivity of 100%, 85% and 67% for PCR, SPN-blot and CPS-CIE, respectively, against culture.

**Table 1. Detection of *S. pneumoniae* by CPS-CIE, SPN-blot and PCR in 65 sputum samples**

<table>
<thead>
<tr>
<th>Possible patterns for detection of <em>S. pneumoniae</em></th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CPS-CIE</strong></td>
<td><strong>SPN-blot</strong></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>
DETECTION OF PNEUMOLYSIN IN SPUTUM

Fig. 1. (a) SDS-PAGE of sputum samples: 10% gel stained with Coomassie Brilliant Blue R-250. Lane M, low mol. wt standards (kDa, BioRad); lanes 1–3, pneumococcal culture-positive samples; 4–6, pneumococcal culture-negative samples. (b) SPN-blot of samples shown in (a). Pneumolysin was detected as single band at c. 53 kDa with a murine MAb to recombinant pneumolysin. Lane M, low mol. wt pneumolysin; 4, 5, negative for pneumolysin; 6, positive for pneumolysin.

Fig. 2. PCR amplification of the pneumolysin gene from sputum samples with a S. pneumoniae primer set: positive band 518 bp, internal control band 300 bp. Lane M, 123-bp ladder (Sigma); lanes 2, 4, 5, sputum samples positive for pneumolysin and internal control; 1, 3, sputum samples negative for pneumolysin but positive for internal control indicating no inhibition; 6, internal control alone (amplification control); 7, reagent blank.

The 38 culture-negative samples included 11 samples from patients receiving antibiotics and from whom S. pneumoniae had been isolated within the previous 2–10 days. PCR and SPN-blot were both positive for six samples, two of which were also positive by CPS-CIE. PCR alone was positive in a further one of these 11 samples and SPN-blot was the only positive test in another.

Assessment of the sensitivity and specificity of the results of the three non-cultural techniques for the remaining 27 S. pneumoniae culture-negative samples (all from patients with no known antecedent pneumococcal infection) can only be in comparison to each other. Eleven of these samples were obtained from patients not receiving antibiotics and all three tests were negative for eight of these. PCR alone was positive for two of these samples and PCR and CPS-CIE (but not SPN-blot) were positive for one sample. Of the remaining 16 S. pneumoniae culture-negative samples, all from patients receiving antibiotics at the time of sampling, all three tests were negative for five samples. PCR was positive for nine samples, six of which were positive by SPN-blot and three of which were also positive by CPS-CIE. CPS-CIE and SPN-blot (but not PCR) were positive for one other sample and SPN-blot was the sole positive test for one sample.

Discussion

We believe that this is the first report of the detection of pneumolysin (SPN) in routine clinical material, although its presence has been noted in pneumococcal exudates in experimental infections [6].

The results of this study show that SPN was detectable by immunoblotting (SPN-blot) in the majority (85%) of sputum samples from which S. pneumoniae was cultured as either pure or predominant growth. SPN-blot is not as sensitive as PCR targeting the pneumolysin gene (SPN-PCR), which was positive for all culture-positive samples, but it is considerably more sensitive than detection of pneumococcal capsular polysaccharide antigen by CIE, which was positive for 67% of the same samples.

The interpretation of the results of the three non-cultural tests on the 38 S. pneumoniae culture-negative samples can only be speculative, as these samples are necessarily heterogeneous, having only their S. pneumoniae culture-negative status in common. Nonetheless, the finding that all three tests were negative for eight of 11 S. pneumoniae culture-negative specimens
from patients not receiving antibiotics and that SPN-blot was negative for all these specimens suggests that the negative non-cultural tests, and in particular SPN-blot, reflect the absence of pneumococcal infection in these samples. This preliminary study did not seek to relate the results of the non-cultural methods to the number of pneumococci in the clinical material, except in a crude semi-quantitative manner. The positivity of culture, PCR and CPS-CIE can be expected to be related directly to the number of pneumococci, whereas SPN-blot, measuring a pneumococcal product which is associated with pneumococcal inflammation, might be more likely to be positive in true infection (when SPN might accumulate) and more likely to be negative in carriage (when the smaller numbers or organisms might not produce enough SPN for detection).

It is also interesting to note that various combinations of positive non-cultural tests were obtained for 11 of the 16 \textit{S. pneumoniae} culture-negative samples from patients receiving antibiotics and with no proof of previous pneumococcal infection. The pattern seen was similar to that found for the 11 samples from patients receiving antibiotics but in whom culture was known to have been recently positive and in which it can be assumed that the positive non-cultural tests reflect the resolving antecedent pneumococcal infection. The differential rates of decline of the three targets (DNA, SPN and CPS) in specimens taken from patients with pneumococcal infections undergoing antibiotic therapy are as yet unknown.

Further studies will be necessary to address many of these issues. In particular, quantitative cultures to permit the distinction between pneumococcal infection and carriage will be essential in the evaluation of any non-cultural test.

The format of the method for SPN immuno-detection also will require considerable revision. Immunoblotting is a labour-intensive, subjective and time-consuming technique and is, therefore, unsuitable for the regular examination of large numbers of routine specimens. Adaptation of immuno-detection of SPN to an ELISA format will greatly facilitate the method, allow quantitation (enhancing the ability to distinguish between infection and carriage) and, in all likelihood, increase the sensitivity. It is also a much more objective method.

Nonetheless, this preliminary study suggests that immuno-detection of pneumolysin in sputum is a promising new approach to the non-cultural diagnosis of pneumococcal chest infection and one which might result in a robust, rapid and affordable method suitable for routine examination of sputum samples.

References