Use of a multiplexed immunoassay for detection of serotype-specific *Streptococcus pneumoniae* antigen in pleural fluid and cerebrospinal fluid specimens

We recently described a method for detection of *Streptococcus pneumoniae* serotype-specific antigens in urine (Sheppard *et al.*, 2011). Here, we report the use of this method for detection of serotype-specific antigen directly from cerebrospinal fluid (CSF) and pleural fluid samples. The use of non-culture-based methods for detection and typing of pneumococci in severe invasive disease cases such as meningitis and empyema is of great benefit since many of these specimens are culture-negative (Balfour-Lynn *et al.*, 2005; Ceyhan *et al.*, 2010). PCR assays have been used in various studies for determining serotypes from isolates (Lawrence *et al.*, 2003) and clinical samples (Morais *et al.*, 2007; Tarragó *et al.*, 2008) but these methods can be complicated and time-consuming to perform and do not give a direct detection of expressed polysaccharides.

Antigen detection using the BinaxNOW immunochromatographic kit [testing for C-polysaccharide (C-Ps)] has recently been shown to be useful for detecting pneumococci in pleural effusions (Casado Flores *et al.*, 2010) and was already validated for use in CSF (Samra *et al.*, 2003). However, this method, whilst useful for primary diagnosis, gives no serotype information, which is a pre-requisite for national surveillance of serious pneumococcal infections and is essential for ascertaining vaccine effectiveness in individual vaccinees.

The use of an ELISA method for serotype-specific antigen detection on pleural fluid samples was reported some time ago (Eltringham *et al.*, 2003). This study showed that the majority of the samples tested were of serotype 1, but the method has not been routinely used due to the large sample volume and considerable reagent requirement for multiple serotype detection using such ELISA methods. The development of our multiplex immunoassay reduces both the complexity and large sample volume requirement for

serotype-specific antigen detection and it is therefore applicable to samples with limited volume, especially those which are inherently difficult to obtain in large volume, such as CSF. We describe in this correspondence the use of the previously described multiplex immunoassay, which detects 14 serotypes/groups, on both pleural effusion and CSF samples for detection of serotypes/groups 1, 3, 4, 5, 6A/C, 6B, 7F/A, 8, 9V, 14, 18, 19A, 19F, 23F and C-Ps.

The assay was performed as previously described (Sheppard *et al.*, 2011); the only deviation was the processing of the CSF and pleural fluid samples prior to testing. To render the samples safe and suitable for testing, the samples were processed in the following way.

CSF samples were diluted 1:3 in sterile PBS (HPA media services) to provide an appropriate volume for processing. Proteinase K (Qiagen) was added at a concentration of 30 mAU ml⁻¹, i.e. 10 µl neat solution in 200 µl diluted CSF or neat pleural fluid sample. The samples were incubated at 56 °C for 10 min on a heating block (Grant) and then transferred to a second heating block set at 95 °C for 30 min to inactivate the proteinase K and any potential pathogens. The samples were then centrifuged at 16 000 g for 2 min. Digested CSF supernatant was removed to a fresh tube, and an aliquot of the digested pleural fluid sample was diluted in PBS to achieve a final 1:5 dilution, ready for testing. Depending on the sample size, up to 100 µl per well of the processed sample was tested in the assay as described previously (Sheppard *et al.*, 2011).

Samples for testing

Since September 2006, the Respiratory and Systemic Infection Laboratory (RSIL) has been testing *S. pneumoniae* PCR-positive CSF and pleural/empyema fluids received from collaborators at the HPA

Meningococcal Reference Unit (Manchester) and Great Ormond Street Hospital (London) in the multiplex immunoassay. The samples were tested at the originating laboratory by specific autolysin PCR assay (Carvalho *et al.*, 2007; Harris *et al.*, 2008) and those received from Manchester HPA were also positive by pneumolysin PCR (Corless *et al.*, 2001). This is part of enhanced surveillance activities aimed at improving information regarding serotype distributions in these, often culture-negative, cases. The serotype result of any matching culture isolates obtained from the patients was only seen when the results of the non-culture assay were added to the laboratory information management system (LIMS). Therefore, the tests were done blinded to any previously obtained serotype results.

In the first 3 years of this enhanced surveillance activity, 323 pneumococcal PCR-positive CSF samples from 300 patients of all ages (33 % below age 17, range 0–86 years) and 223 pneumococcal PCR-positive pleural/empyema fluid samples from 214 children up to the age of 16 were received from the sending centres, who were asked to send all *S. pneumoniae* PCR-positives on for testing in the multiplex assay. All samples received at RSIL are included in this analysis. Retrospective review of LIMS data revealed that serotype data from *S. pneumoniae* isolates were only obtained from 79 (26 %) of the patients from whom a CSF sample was received and 8 (4 %) of the patients from whom a pleural fluid sample was received.

Results obtained from the samples

Serotypes were determined directly from 176 CSF samples (54 %), and an additional 79 (24 %) were positive for pneumococcal C-Ps only. In total, 139 of the 300

meningitis patients had serotypes determined directly from the CSF in the absence of a culture isolate. Assay serotypes were detected in 207 (93 %) of the pleural fluid samples, and an additional 9 (4 %) were positive for pneumococcal C-Ps only. This added serotype determination for 199 of the 214 pleural effusion patients from whom a culture isolate was not available. The number and distribution of the serotypes detected are shown in Table 1.

For those where a matching culture isolate was available (94 from 87 patients), all clinical specimens gave the expected serotype results (i.e. serotype-positive where the serotype was available in the assay and serotype-negative where another serotype was isolated), except two CSF samples that had matched cultures serotyped as 14 and 7F and were negative for serotype in the assay. Therefore, the sensitivity for assay types was 97 % for CSF samples and 100 % for pleural fluid samples against culture. Specificity was 100 % for both sample types against culture.

In general, the very high signal strengths obtained from the pleural fluid specimens suggested an abundance of polysaccharide present in the samples (the results were

indicative of >50 ng polysaccharide ml^{-1} in many cases). In comparison, the strengths of the signals from CSF samples tended to vary more greatly in concentration from sample to sample but with many <0.5 ng ml^{-1} . There was a noticeably different distribution of serotypes present in the pleural fluid specimens compared to the CSF specimens, with serotypes 1 and 3 being the predominant serotypes in pleural fluid specimens, supporting the findings of previous studies (Tarragó *et al.*, 2008), and a much more diverse range of serotypes present in the CSF specimens, which was more similar to the overall distribution of serotypes from all age groups seen in the culture data (Table 1).

The samples positive only for the C-Ps suggest the presence of serotypes not detectable in the assay. Some of the PCR-positive specimens were completely negative in the assay. There were more negative results from the CSF samples where the overall signal strengths of the positive samples were much lower; this may be due to very small amounts of bacteria present and in some cases very small amounts of sample (<5 μl) available to test. Negative results

may also be due to sensitivity issues with the C-Ps assay, as discussed in the original paper, where it was found that not all samples, even when positive for a serotype, were also positive for C-Ps. False-positives in the PCR assays are another possible source of the negative results, although this is unlikely due to the targets selected for PCR testing.

Conclusions

The use of the previously described multiplex immunoassay on CSF and pleural fluid samples has been shown to be a valuable source of serotype information for enhanced surveillance activities and conjugate vaccine effectiveness estimates which would otherwise not be available. The assay performs particularly well on pleural fluid samples, partly due to the distribution of serotypes (more of those available in the assay) and partly due to the large amount of polysaccharide detectable in these specimens. The small sample volume requirement of the assay makes it ideal for detection of serotype directly from CSF where a culture has not grown. In some cases, positive signals from serotypes were obtained from samples with less than 5 μl CSF volume remaining.

This assay will be a valuable tool for surveillance of pneumococcal serotype distributions in severe invasive diseases in the era of conjugate vaccines. Provided that the laboratory performing the assay has the appropriate equipment and reagents are available, the cost per test is less than that of conventional culture and serotyping by traditional methods.

Carmen L. Sheppard,¹ Malcolm Guiver,² John Hartley,³ Timothy G. Harrison¹ and Robert C. George¹

¹Respiratory and Systemic Infection Laboratory, Health Protection Agency – Microbiology Services Division Colindale, 61 Colindale Avenue, London NW9 5EQ, UK

²Health Protection Agency North West, Manchester Laboratory, Manchester Medical Microbiology Partnership, PO Box 209, Clinical Sciences Buildings, Manchester Royal Infirmary, Oxford Road, Manchester M13 9WL, UK

³Department of Microbiology, Virology and Infection Control, Level 4, Camelia Botnar Laboratories, Great Ormond

Table 1. Number and percentage of serotypes detected directly from CSF and pleural fluid specimens September 2006–August 2009, and percentages seen in all sterile site isolates from the UK [pneumococcal culture serotyping data over the same time period (13 720 isolates)]

Serotype	Direct detection from CSF [<i>n</i> (%)]	Direct detection from pleural fluid [<i>n</i> (%)]	Sterile site culture data (%)
1	17 (5.3)	106 (47.5)	11.4
3	32 (9.9)	54 (24.2)	6.9
4	9 (2.8)	1 (0.4)	3.9
5	1 (0.3)	10 (4.5)	0.7
6A/C	9 (2.8)	1 (0.4)	5.1
6B	14 (4.3)	3 (1.3)	3.6
7F/A	20 (6.2)	12 (5.4)	8.5
8	9 (2.8)	2 (0.9)	7.5
9V	6 (1.9)	3 (1.3)	5.0
14	9 (2.8)	5 (2.2)	6.6
18	11 (3.4)	0 (–)	2.4
19A	19 (5.9)	9 (4.0)	5.8
19F	11 (3.4)	0 (–)	3.1
23F	9 (2.8)	1 (0.4)	3.9
C-Ps +ve only*	79 (24.5)	9 (4.0)	–
Negative	68 (21.1)	7 (3.1)	–
Total	323	223	

*C-Ps, C-polysaccharide.

Street Hospital, Great Ormond Street,
London WC1N 3JH, UK

Correspondence: Carmen L. Sheppard
(carmen.sheppard@hpa.org.uk)

Balfour-Lynn, I. M., Abrahamson, E., Cohen, G., Hartley, J., King, S., Parikh, D., Spencer, D., Thomson, A. H., Urquhart, D. on behalf of the Paediatric Pleural Diseases Subcommittee of the BTS Standards of Care Committee (2005). BTS guidelines for the management of pleural infection in children. *Thorax* **60** (Suppl. 1), i1–i21.

Carvalho, M. G., Tondella, M. L., McCaustland, K., Weidlich, L., McGee, L., Mayer, L. W., Steigerwalt, A., Whaley, M., Facklam, R. R. & other authors (2007). Evaluation and improvement of real-time PCR assays targeting *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. *J Clin Microbiol* **45**, 2460–2466.

Casado Flores, J., Nieto Moro, M., Berrón, S., Jiménez, R. & Casal, J. (2010). Usefulness of pneumococcal antigen detection in pleural effusion for the rapid diagnosis of infection by *Streptococcus pneumoniae*. *Eur J Pediatr* **169**, 581–584.

Ceyhan, M., Yildirim, I., Sheppard, C. L. & George, R. C. (2010). Pneumococcal serotypes

causing pediatric meningitis in Turkey: application of a new technology in the investigation of cases negative by conventional culture. *Eur J Clin Microbiol Infect Dis* **29**, 289–293.

Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Fox, A. J. & Kaczmarek, E. B. (2001). Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. *J Clin Microbiol* **39**, 1553–1558.

Eltringham, G., Kearns, A., Freeman, R., Clark, J., Spencer, D., Eastham, K., Harwood, J. & Leeming, J. (2003). Culture-negative childhood empyema is usually due to penicillin-sensitive *Streptococcus pneumoniae* capsular serotype 1. *J Clin Microbiol* **41**, 521–522.

Harris, K. A., Turner, P., Green, E. A. & Hartley, J. C. (2008). Duplex real-time PCR assay for detection of *Streptococcus pneumoniae* in clinical samples and determination of penicillin susceptibility. *J Clin Microbiol* **46**, 2751–2758.

Lawrence, E. R., Griffiths, D. B., Martin, S. A., George, R. C. & Hall, L. M. (2003). Evaluation of semiautomated multiplex PCR assay for

determination of *Streptococcus pneumoniae* serotypes and serogroups. *J Clin Microbiol* **41**, 601–607.

Morais, L., Carvalho, M. G., Roca, A., Flannery, B., Mandomando, I., Soriano-Gabarró, M., Sigauque, B., Alonso, P. & Beall, B. (2007). Sequential multiplex PCR for identifying pneumococcal capsular serotypes from South-Saharan African clinical isolates. *J Med Microbiol* **56**, 1181–1184.

Samra, Z., Shmueli, H., Nahum, E., Paghis, D. & Ben-Ari, J. (2003). Use of the NOW *Streptococcus pneumoniae* urinary antigen test in cerebrospinal fluid for rapid diagnosis of pneumococcal meningitis. *Diagn Microbiol Infect Dis* **45**, 237–240.

Sheppard, C. L., Harrison, T. G., Smith, M. D. & George, R. C. (2011). Development of a sensitive, multiplexed immunoassay using xMAP beads for detection of serotype-specific *Streptococcus pneumoniae* antigen in urine samples. *J Med Microbiol* **60**, 49–55.

Tarragó, D., Fenoll, A., Sánchez-Tatay, D., Arroyo, L. A., Muñoz-Almagro, C., Esteva, C., Hausdorff, W. P., Casal, J. & Obando, I. (2008). Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. *Clin Microbiol Infect* **14**, 828–834.