Detection of pneumolysin in cerebrospinal fluid for rapid diagnosis of pneumococcal meningitis

Reba Kanungo, M. Bhaskar, A. Kumar, S. Badrinath & B. Rajalakshmi

Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education & Research Pondicherry, India

Received October 21, 2002

Background & objectives: Pneumolysin, a toxin produced by Streptococcus pneumoniae is associated with virulence and is found in all invasive isolates. Its role as a diagnostic tool has recently been exploited. Most of the methods used are based on molecular techniques and are not cost-effective. The present study was undertaken to evaluate a simple, rapid and cost-effective method to detect pneumolysin in CSF as a diagnostic test for pneumococcal meningitis.

Methods: A total of 75 CSF samples from children with presumptive diagnosis of acute pyogenic meningitis or encephalitis were subjected to Gram stain, culture and pneumolysin detection by Cowan 1 staphylococcal protein A co-agglutination technique.

Results: Pneumolysin was detected in 26(78.8%) of 33 culture proven CSF samples and 4(9.5%) of 42 culture negative samples. Antigen detection by Co-A had a specificity of 90 per cent and a sensitivity of 79 per cent when compared with culture. Compared to Gram stain, pneumolysin Co-A had a specificity and sensitivity of 91.0 and 92.0 per cent respectively.

Interpretation & conclusion: Detection of pneumolysin was found to be a simple, low cost antigen detection assay for rapid diagnosis of pneumococcal meningitis, for routine use in the developing countries.

Key words CSF - co-agglutination - pneumolysin - pneumococcal meningitis

Streptococcus pneumoniae continues to be a major cause of morbidity and mortality as it causes meningitis and pneumonia in paediatric and old populations. Despite treatment, case fatality rates are alarmingly high in the developing countries. Successful management of meningitis depends on rapid confirmatory diagnosis. Rapid and sensitive methods of detecting capsular polysaccharide antigens by latex agglutination, immunoelectrophoresis and coagglutination have been in use for a very long time1-4. However, dependence on serotype or sero group causing infection has been one of the limiting factors5.

Haemolysins produced by streptococci and staphylococci have been used in antigen detection assays by neutralization and enzyme immunoassays6. Pneumolysin, produced by all invasive strains of pneumococci, is a species-specific 53 kD, thiol activated protein7. It is associated with virulence of the organism and hence is thought to be involved in the pathogenesis of invasive pneumococcal disease8,9. Work has been underway to study the efficacy of pneumolysin as a protein conjugate vaccine with capsular polysaccharide9. Evaluation of diagnostic usefulness of pneumolysin and its corresponding antibody detection in clinical specimens is beginning to receive attention10,11. Most of the methods employed are based on molecular techniques. Recombinant antigens have been developed taking advantage of the protein nature of pneumolysin.
Monoclonal antibodies against the genetically engineered antigen are being used in detecting pneumolysin in clinical specimens. The cost of techniques involved in genetically deriving pure antigen and raising specific monoclonal antibody may be a limiting factor in using these procedures for laboratory diagnosis of pneumococcal infections. The present study was designed to evaluate a simple method to detect pneumolysin in the CSF of children suspected to have pneumococcal meningitis.

**Material & Methods**

Seventy five children with presumptive diagnosis of acute pyogenic meningitis or encephalitis, aged between 1-144 months, admitted during 1998-2000 to the paediatric ward of the Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, a tertiary care hospital in south India, were included in the study. Children with diarrhoea and other neurological involvement were excluded from the study. CSF (0.5-1 ml) collected from these children was subjected to Gram stain, culture and antigen detection test. Gram staining and standard bacteriological procedures were done to detect, isolate and test the antibiotic susceptibility of the organisms. The remaining CSF samples were stored at -70°C till further use.

**Pneumolysin production**: Pneumolysin was prepared according to the method of Kanclerski and Molby, with a slight modification. Briefly, five clinical isolates from CSF were grown in 10 per cent glucose broth overnight at 37°C in a CO2 incubator. Subsequently this was inoculated in 500ml of the same medium to get a larger bacterial volume. The growth was intermittently shaken for increased aeration during incubation. The culture suspension was washed three times with phosphate buffered saline (PBS pH 7.2) and resuspended in 50ml of glucose broth. The suspension was kept at 37°C for 72 h until autolysis occurred which was evident by clearing of the broth. The resultant clear broth was dialysed overnight with PBS, pH 7.4. Protein was precipitated by 60 per cent ammonium sulphate and redissolved in PBS, pH 7.2. This formed the antigen which was used to raise polyclonal antibody in rabbits. Further purification of the antigen was not possible due to lack of necessary equipment. Protein content of pneumolysin was determined by the Lowry’s method.

Haemolytic activity of pneumolysin was determined by sheep cell haemolysis. Briefly, to a series of two-fold dilutions of 25µl of pneumolysin in PBS (pH 7.2), washed fresh sheep red blood cells (RBCs) were added, at a concentration of 2 per cent and incubated at 37°C for 1h and subsequently, overnight at 4°C. The dilution showing complete haemolysis was taken as the end point. The haemolytic titre was determined to be 40HU/ml.

**Polyclonal antipneumolysin test reagent**: Neat pneumolysin was added in a ratio of 1:1 with incomplete Freund’s adjuvant (Sigma, USA) and inoculated into rabbits at a dose of 1ml intramuscularly at weekly intervals for eight weeks. Two weeks after the last dose, the blood was collected and serum was titrated for antipneumolysin by the neutralization test. Doubling dilutions of the rabbit antiserum was made in 25µl volumes in microtitre plates using PBS, pH 7.2 as the diluent. To this an equal volume of neat pneumolysin was added and incubated at room temperature for 1 h. Sheep RBCs (2%) were added to the microwells, incubated for 1 h at 37°C and subsequently over night at 4°C. Dilution of antisera showing highest titre of neutralization was used subsequently for sensitizing staphylococcal Co-A (obtained from the Christian Medical College, Vellore, India) reagent. For preparing the reagent the method of Lalitha et al was followed. Neat antipneumolysin serum (1ml) was added to 1ml of 10 per cent staphylococcal Co-A cells suspension. This was left at room temperature with intermittent shaking. After 30 min the suspension was centrifuged at 800g for 15 min and resuspended in 5 ml of PBS and stored at 4°C till further use.

**Pneumolysin Co-A test procedure**: To 20 µl of CSF sample on a clean slide, equal volume of Co-A reagent was added and rotated for one min. Positive test was indicated by clumping of the staphylococcal cells with clearing of the background. Unsensitised Cowan I staphylococcal cell suspension was used as negative control with every test. Sensitivity and specificity of the test was calculated with reference to that of culture and Gram stain.

The study protocol was approved by the ethics committee of the institution.

**Results**

Of the total 75 CSF samples tested, 33 were positive for S. pneumoniae by culture, and of these 33, 26 had...
detectable pneumolysin, giving a sensitivity of 79 per cent (Table I). Thirty eight out of 42 culture negative samples were negative for pneumolysin while four of culture negative samples were positive for pneumolysin, giving the test a specificity of 90 per cent. Pneumolysin detection was also compared with Gram stain and found to have a sensitivity of 92.0 per cent and specificity of 91.0 per cent with a positive predictive value of 86.6 per cent and negative predictive value of 95.3 per cent (Table II).

Antibiotic susceptibility test showed all isolates to be sensitive to penicillin (by oxacillin screening), ampicillin, cefotaxime and ciprofloxacin.

**Table I.** Pneumolysin detection in CSF by co-agglutination test compared with culture

<table>
<thead>
<tr>
<th>Culture positive</th>
<th>Culture negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-A Pneumolysin +</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Co-A pneumolysin -ve</td>
<td>7</td>
<td>38</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>42</td>
</tr>
</tbody>
</table>

Sensitivity : 79%
Specificity : 90%
Positive predictive value : 86.6%
Negative predictive value : 84.4%
Efficiency : 85.3% (95% CI = .65-.91)

**Table II.** Pneumolysin detection in CSF by co-agglutination test compared with Gram stain

<table>
<thead>
<tr>
<th>Gram smear positive</th>
<th>Gram smear negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-A Pneumolysin +</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Co-A pneumolysin -ve</td>
<td>2</td>
<td>43</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>47</td>
</tr>
</tbody>
</table>

Sensitivity : 92%
Specificity : 91%
Positive predictive value : 86.6%
Negative predictive value : 95.3%
Efficiency : 92% (95% CI = .85-.97)

**Discussion**

Pneumolysin is a multifunctional pneumococcal virulence factor that appears to facilitate growth and dissemination during the early stages of pneumococcal infections. Detection of pneumolysin would therefore lead to an early diagnosis of the infection. In the present study pneumolysin was detected in 26 of the 33 culture positive CSF, with a sensitivity of 79 per cent. Negative result in 42 culture negative samples gave the test a specificity of 90 per cent. Culture results are often determined by prior antibiotic therapy. Pneumolysin released from the organism is not affected by the antibiotics, hence its detection may have an advantage in culture negative cases.

Detection of pneumolysin, which is released by all invasive strains of pneumococci, has a distinct advantage over capsular polysaccharide antigen detection, since the latter shows some cross-reaction with other carbohydrate antigens. Presence of pneumolysin reflects an increased virulence suggesting the lysis of cells and release of toxin. Antibiotics in this case can no longer contain the progression of the disease thereafter.

Various methods for the detection of pneumococcal capsular polysaccharide antigen from clinical specimens have been evaluated. Polymerase chain reaction (PCR) assays to detect the pneumolysin DNA from middle ear fluid in otitis media, and from sera of children with pneumococcal pneumonia have been reported. Pneumolysin in the urine of children with pneumococcal pneumonia has also been evaluated. A rapid PCR based detection of S. pneumoniae DNA from CSF has also been reported by Kearns et al. Most of the tests are expensive when compared to co-agglutination which is a simple technique that can be performed as a rapid bedside test. It has all the advantages of a particle agglutination test and is simple to interpret with an added advantage of being inexpensive. The reagents can be prepared in the laboratory. Shelf life of the sensitised co-agglutination reagent was found to be six months when stored at 4°C.

In conclusion, the present study suggests that the detection of pneumolysin in CSF by co-agglutination is a promising new technique for rapid diagnosis of pneumococcal meningitis, and is affordable for routine use in the developing countries. Purification of pneumolysin and use of monoclonal antibodies may further improve its efficacy as an antigen detection system.
References


Reprint requests : Dr Reba Kanungo, Professor and Head, Department of Clinical Microbiology Pondicherry Institute of Medical Sciences, Kalapet, Pondicherry 605014, India e-mail : rebakanungo2001@yahoo.com