Serotyping of *Streptococcus pneumoniae* by agglutination assays: a cost-effective technique for developing countries

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There is a need for additional data on the distribution of pneumococcal serotypes in developing countries. We report the use of a coagglutination (COA) and a latex agglutination (LA) test for serotyping *Streptococcus pneumoniae* which were evaluated using 114 clinical isolates in Vellore, India. In tests to serotype 30 fresh isolates of pneumococci from meningitis (8 isolates), bacteraemia/septicaemia (21 isolates) and peritonitis (1 isolate) cases, there was complete concordance among the three methods. An additional 20 isolates (11 from cerebrospinal fluid and 9 from blood cultures) were serotyped using both LA and COA, with full agreement between the results. With a further 30 isolates, there was 93% concordance for the COA types with serotypes assigned by a WHO reference laboratory. The COA and LA serotyping results were equivalent in accuracy to those obtained using quellung serotyping. Both these agglutination tests are rapid, valid, and relatively cheap, and with appropriate validation by reference laboratories they could be more widely used in developing countries to obtain local and regional data on pneumococcal serotype distribution.

Introduction

Invasive pneumococcal diseases are a major cause of morbidity and mortality, especially in developing countries, and are the focus of attention for prevention by immunization (1, 2). The currently available vaccines are serotype-specific and include those serotypes identified as important according to their relative frequency in surveillance studies (3). Since the distribution of pneumococcal serotype varies over time in a given location (4) as well as between locations (2, 5–8) and age groups (9, 10), locally obtained surveillance data are essential in order to understand local epidemiology and to plan national or regional vaccine strategies. The need for additional data on pneumococcal serotype distribution from developing regions, especially in Asia, has been noted (2, 9).³

The standard technique for determining pneumococcal serotype is the quellung reaction, which requires relatively expensive reagents and is labour intensive. We have evaluated prospectively the utility and cost of modified coagglutination and latex agglutination tests compared with the quellung reaction for serotyping of pneumococci in a clinical microbiology laboratory in India and report our results in this article.

Materials and methods

*Pneumococci and antisera.* Investigated were pneumococcal strains isolated from normally sterile sites identified using standard microbiological techniques at the Department of Microbiology, Christian Medical College Hospital, Vellore, from December 1992 to September 1994. The tests described below used 0.5 ml of a saline suspension of overnight growth of pneumococci on trypticase soy blood agar (TSBA) incubated in a candle extinction jar. The opacity of the bacterial suspension was adjusted to

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McFarland standard 1. The antisera used for all the serotyping methods were obtained from Statens Seruminstitut, Copenhagen, Denmark.

**Quellung reaction.** Approximately 20μl of the culture suspension, as prepared above, was placed on a new, clean, scratch-free glass slide and a “spot smear” made. The smear was air dried and then heat fixed. Samples of antiserum (10μl) from pools A–I were added to the correspondingly marked smears, together with 10μl of methylene blue and the slide mounted with a coverslip. The smears were allowed to stand for 10–15 minutes and then examined using an oil-immersion objective. A positive reaction was taken to be a well-defined capsular swelling at ×1000 magnification. The test was repeated with individual serogroup/type-specific sera.

**Latex agglutination.** The latex agglutination (LA) test, which we previously developed and standardized (8), was modified for serotyping isolates by incorporating all pool and serogroup/type-specific reagents, rather than a limited number of serotypes. The latex particle suspension was obtained from Interfacial Dynamics Corporation, Portland, OR, USA.

The LA test was performed on clear glass slides with 14-mm diameter ceramic rings, by placing 25μl of the culture suspension (prepared as for the coagglutination (COA) test), 10μl of a chaotrophic buffer (GBS-BSA with 0.2mol/l urea and 4% polyethylene glycol 8000) and 10μl of the latex reagent and rotating the plate at 120rpm at room temperature for 10min in a moist chamber. The reactions were immediately read against a background light. A positive test showed aggregation of latex particles and clearing of the suspension.

**Coagglutination.** Coagglutination reagents were prepared as described previously (11, 12). The COA test was carried out on a glass slide with 14-mm diameter ceramic rings. Each of the pool sera reagents (25μl) was added to 25μl of the culture suspension. The slide was rotated by hand for 2min and the results read with the naked eye. The test was interpreted as positive if clumping of cells and clearing of the suspension occurred within 2min.

**Results**

A total of 30 clinical isolates of pneumococci (21 from blood, 8 from cerebrospinal fluid (CSF) and one from peritoneal fluid) were used to compare the three serotyping techniques. The serotypes identified by the quellung reaction included the following: type 1 (nine isolates); type 5 (four isolates); types 6, 7, and 19 (three isolates each); type 20 (two isolates); and types 3, 10, 11, 12, 13, and 34 (one isolate each). Identical serotypes were assigned using all three serotyping methods. Further comparisons were made of the results of the COA test and the LA procedure. For this purpose 20 additional clinical isolates (11 from CSF and 9 from blood cultures — type 1 (nine isolates), type 5 (three), types 38 and 6 (two isolates each) and types 4, 12, 19, and 23 (one each)) were serotyped using both LA and COA techniques. Complete concordance was observed. For all three methods studied there was no cross-reactivity among the serogroups/types within the recognized pool.

We have employed the COA technique for routine serotyping, including 64 isolates of pneumococci from patients admitted with invasive disease who were enrolled as part of an ongoing invasive bacterial infections study (IBIS). These included 21 isolates from CSF, 32 from blood, and 11 from fluid/pus cultures. A total of 30 of these isolates were sent as coded lyophilized cultures to the WHO Collaborating Centre for Reference and Research on Pneumococci, Statens Seruminstitut, Copenhagen, for blind confirmatory serotyping. The level of concordance of the assigned serotype between the two laboratories was 28/30 (93%). The concordant strains included eight type 1, three each of type 6 and 12, two each of types 3, 19, and 35, and one each of types 4, 7, 14, 15, 20, 21, and 33. Two strains assigned serotype 4 and 38 in Vellore were assigned type 6B and 19A, respectively, in Copenhagen, and an isolate positive only in pool G was identified as type 35B. Overall, there was agreement for 58/60 (97%; 95% confidence limits: 89–100%) of the strains tested independently and blind using both the quellung and COA techniques.

**Discussion**

Of the various tests available for serotyping of pneumococci, the quellung reaction, first described in 1902 by Neufeld (13), has remained the reference method. Other serotyping techniques, e.g., counter-immunoelectrophoresis (CIE) (14), LA (15), and a checkerboard technique utilizing 12 pooled antisera (16), have been described. The COA method for serotyping of pneumococci was first developed and used by Kronvall (11), who reported that in 17 of 89 pneumococci it exhibited weak cross-reactions with one or more capsular serotypes in addition to the quellung serotype. A commercial COA test kit (Phadebact, Piscataway, NJ, USA) has been evaluated (17) for identification of pneumococci, but
not for serotyping, since it uses the omniserum reagent alone.

Our findings confirm the high utility and accuracy of the LA and COA tests for serotyping pneumococci isolated from individuals with invasive disease. The reactions were strong, agglutination with clearing being observed within seconds. Use of TSBA and fresh culture suspensions made from the primary culture plates probably contributed to the accuracy, as shown by the high concordance with typing in the WHO Collaborating Centre.

The quellung reaction requires undiluted antisera, which accounts for its relatively high cost per test (Table 1), as well as a substantial amount of technician’s time, since the results of all the reactions must be inspected with a microscope. In comparison with the other two serotyping techniques studied, COA has the advantage of being quicker. The LA test makes use of diluted antisera, although the latex particle suspension is relatively expensive. Use of Cowan I Staphylococcus aureus as the COA reagent makes the test economical for most laboratories in developing countries. The essential immunological reagent for all tests is rabbit antiserum (available in 1994 from Statens Seruminstitut, for approximately US$ 4088 for a complete set of 9 pool antiserum (each reacting with 7–11 serotypes)) and 46 type or group antiserum. These expensive reagents are used more efficiently in the COA technique because the protein-A-producing Cowan I S. aureus specifically binds the Fc portion of the antibodies, orienting the Fab moiety outwards; this favourable orientation allows lower concentrations of the antisera to be used in preparing the reagent. Preparation of LA reagents requires use of a high speed centrifuge, whereas COA reagents can be prepared, in larger volumes, using a standard centrifuge. Once prepared, COA and LA reagents can be stored refrigerated for up to 1 year without loss of potency.

The modified COA and LA tests proved to be rapid, accurate, valid and cost-effective methods, which can be readily introduced to microbiology laboratories for surveillance of pneumococci.

Careful training and supervision of technical personnel, with meticulous quality control procedures and periodic validation of serotypes with a reference laboratory, are essential for successful local serotyping irrespective of the technique used (18). Isolates should be stored lyophilized or at −70°C so that they are available for subtyping within groups, and for assessment of other antigens by reference laboratories (2).

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Résumé
Determination des sérotypes de Streptococcus pneumoniae au moyen d’une épreuve d’agglutination: une technique de bon rapport coût-efficacité pour les pays en développement
Il est nécessaire de disposer de plus amples données sur la distribution des sérotypes de pneumocoques dans les pays en développement. La technique standard de typage sérologique (réaction de Neufeld) étant coûteuse et laborieuse, nous avons mis au point deux autres techniques – une épreuve de coagglutination (COA) et une épreuve d’agglutination sur latex (LA) – pour la détermination des sérotypes de Streptococcus pneumoniae, et les avons évaluées sur 114 isoléments cliniques recueillis à Vellore (Inde). Sur 30 isoléments frais de pneumocoques provenant de cas de méningite (8 isolements), de bactériémie/septicémie (21 isolements) et de péritonite (1 isolément), la concordance était totale entre les trois méthodes. Vingt autres isoléments (11 de liquide céphalorachidien et 9 d’hémocultures) ont été typés par LA et COA, avec un accord total entre les résultats obtenus avec ces deux méthodes. Sur 30 autres isoléments, la concordance était de 93% entre les sérotypes obtenus par COA et ceux obtenus par un laboratoire de référence de l’OMS. Les résultats étaient d’une exactitude équivalente à

Table 1: Characteristics and estimated costs of reagents for the serotyping techniques used in the studya

<table>
<thead>
<tr>
<th>Method</th>
<th>Test reagent volumea</th>
<th>Antiserum dilution</th>
<th>Reagent cost per serotyping (US $)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quellung</td>
<td>10 µl</td>
<td>None</td>
<td>15.0</td>
</tr>
<tr>
<td>Latex agglutination</td>
<td>10 µl</td>
<td>1:200</td>
<td>0.25</td>
</tr>
<tr>
<td>Coagglutination</td>
<td>25 µl</td>
<td>1:100</td>
<td>0.20</td>
</tr>
</tbody>
</table>

a Labour or personnel costs are not included in the cost estimates.

b Volume per serotyping assay.

c Assuming that nine pools and six type/group tests are needed to assign a serotype to one unknown strain.

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ceux obtenus par la réaction de Neufeld. Les épreuves COA et LA sont rapides, valables et relativement peu coûteuses car les immunsérum étafons utilisés pour le typage peuvent être utilisés à des dilutions élevées. L’utilité et l’exactitude de ces épreuves laissent à penser qu’avec une validation appropriée par des laboratoires de référence, elles pourraient être plus largement utilisées dans les pays en développement pour l’obtention de données locales et régionales sur les sérotypes de pneumocoques.

References