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Evaluation of a new serotyping kit for *Streptococcus pneumoniae*

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A new serotyping test kit (*Streptococcus pneumoniae* antisera 'Seiken' set; Denka kit) was evaluated for 285 strains of *Streptococcus pneumoniae* in comparison with the standard capsular reaction (Quellung test). This new kit is based on the slide-agglutination method and is composed of eight pool sera, 40 group or type sera and 41 specific type sera. All serotyping results by using the Denka kit were completely identical to those obtained by using the conventional Quellung test. For types and groups, sensitivity and specificity were 100 and 100 %, respectively. For specific types, sensitivity and specificity were 100 and 100 %, respectively. The Denka kit is relatively rapid (mean test time, 5 min, versus 15 min by Quellung test), cheap (0·5 US$ per test, versus 1·4 US$ per Quellung test), easy to perform and does not require special equipment. The Denka kit may be useful for fieldworkers in developing countries involved in epidemiological surveys and vaccine development.

INTRODUCTION

Streptococcus pneumoniae is a major community-acquired pathogen that causes serious pneumonia, otitis media, septicaemia and meningitis worldwide (Roberts, 1979). In adults, types 1–8 are responsible for about 75 % of pneumococcal pneumonia cases and for more than half of pneumococcal bacteraemia cases, whereas in children, types 6, 14, 19 and 23 are frequently responsible (Brooks *et al.*, 1998). Infections caused by *S. pneumoniae* can be life-threatening, particularly to children aged < 2 years; the highest risk is to children aged 0–11 months and elderly people (Cant, 2002).

Pneumococcal vaccines are made from purified capsular polysaccharides. More than 90 capsule types and serotypes are known in *S. pneumoniae* (Hausdorff *et al.*, 2002). The polysaccharide vaccine for *S. pneumoniae* (Pneumovaccus^{R}) is composed of 23 different capsule antigens. This vaccine is very useful for prevention of pneumococcal infection in adults. However, in children aged < 2 years, the acquisition rate of immunity to *S. pneumoniae* is insufficient because of low immunogenic activity of the bacterium and premature immunity of the subjects (Hausdorff *et al.*, 2000). Several new conjugated pneumococcal vaccines have been developed, but the antigens selected for these vaccines are limited to only 7, 9 or 11 different serotypes, mainly of penicillin-resistant *S. pneumoniae* (Joloba *et al.*, 2001). Serotyping of *S. pneumoniae* is very important for epidemiological analysis and pneumococcal vaccination.

The capsular reaction (Quellung test) is the gold standard used to determine serotypes of *S. pneumoniae* (Sorensen, 1993). It is very reliable and reproducible, but is also labour-intensive and not easily affordable. Denka Seiken has developed a new method (*Streptococcus pneumoniae* antisera 'Seiken' set; Denka kit) based on slide-agglutination, which is cheap, simple, rapid and reliable. We evaluated this kit for epidemiological surveys of *S. pneumoniae*.

Abbreviations: CIEP, counterimmunoelectrophoresis; COA, coagglutination; LA, latex agglutination.

METHODS

Bacterial strains and culture methods. In total, 285 strains of *S. pneumoniae* were used (130 from Nepal and 155 from Japan). These isolates were stored at −85 °C in 10 % skimmed milk until use. Each strain was maintained on Columbia agar with 5 % sheep blood at 35 °C for 16 h in 5 % CO_2. *S. pneumoniae* was identified by conventional methods, including optochin susceptibility and bile solubility tests (Doern *et al.*, 1999). These identification results were confirmed by PCR amplification of *lytA*, the pneumococcal-specific autolysin gene (Nagai *et al.*, 2001).

Quellung test. The Quellung test was performed as described previously by using anti-pneumococcal capsule serum from the Statens Serum Institute, Denmark (Lund & Henrichsen, 1978; Sorensen, 1993).

Slide-agglutination method for serotyping. The Denka kit is supplied by Denka Seiken. It is intended for determination of *S. pneumoniae* serotypes.

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Sera were prepared by hyperimmunizing healthy rabbits with standard strains of known serotypes of pneumococci, which were obtained from the American Type Culture Collection (ATCC). After bleeding, antisera were separated, heated at 56 °C for 30 min, absorbed to remove cross-agglutinins and sterilized by antibacterial filtration.

The Denka kit is composed of eight pool sera, 40 group or type sera and 41 specific type factor sera. The first kit contains eight vials of polyvalent (pool) antisera and 40 vials of monovalent group or type antisera. The Denka kit covers all vaccine types and several major non-vaccine types of pneumococci (Table 1). Pool sera recognize several antigens, which are serogroups or types within each pool. For example, pool 1 recognizes pneumococcal antigens types 1, 2, 3, 4 and 5, whilst pool 2 recognizes 6, 8, 9 and 10 type or group sera. Similarly, pools 3–8 recognize type or group sera as shown (Table 1). Factor sera work on the same principle, but are more specific; they recognize antigens within a particular serotype or group. The second kit contains 41 specific type factor sera (Table 2).

The Denka kit is used as follows. A single colony is obtained after culturing bacteria. A glass slide is divided into several parts for testing the isolate to be typed. A drop (25 µl) of each antiserum to be tested is placed onto the respective section of the prepared slide. The same is done for a control with normal saline solution. By using a toothpick or sterile microbacteriological loop, a small but visible amount of bacteria is picked and mixed directly with the drop of antiserum, spreading over the partitioned area. The glass slide is then tilted back and forth to look for agglutination (Fig. 1). Agglutination occurs only when bacteria are mixed with specific sera, which react with bacterial antigens to cause an antigen–antibody reaction if the serotype of the isolate is contained within the antiserum being used. Agglutination that occurs within 1 min can be observed visually and is considered to be a positive reaction (Fig. 2). For the control, bacteria are mixed with physiological saline solution to check if agglutination occurs spontaneously. Reactions are performed at room temperature.

In this study, we first used pool sera to determine the pool to which pneumococci belonged by using the procedure described above. Thereafter, bacteria were tested with each type or group serum within the pool; for instance, if the bacteria agglutinated with pool 1 serum, we next picked bacterial colonies and checked them against type or group sera 1, 2, 3, 4 and 5 (Table 1). Finally, the specific type was checked similarly by using specific type factor sera (Table 2). Some types or groups do not have specific types, e.g. 1, 2, 3, 4, 5, 8, 13, 14, 20, 21, 25, 27, 29, 31, 34, 36, 37, 38, 39, 40, 42, 43, 44, 45, 46 and 48. For these types, the procedure of checking for agglutination stopped at the level of serotype or group. For the rest, we determined the specific type by using specific factor sera that recognized certain antigens or antigenic groups with reactions as shown (Table 2). For example, if a bacterial colony formed agglutination with pool 2 serum and then with type 6 serum, if on further reaction it formed agglutination with specific factor serum 6b and not with 6c (6b+ 6c−), then the specific type is 6A; the pattern 6b− 6c+ would indicate specific type 6B. Such reactions were carried out for all bacteria tested and patterns of agglutination were interpreted with the aid of the list provided with the kit. Typical slide-agglutination positive and negative controls are shown in Fig. 2.

We also checked the Denka kit for reaction against 18 type strains of streptococci other than pneumococci: *Streptococcus agalactiae*, *Streptococcus anginosus*, *Streptococcus bovis*, *Streptococcus canis*, *Streptococcus constellatus*, *Streptococcus cristatus*, *Streptococcus dysgalactiae*, *Streptococcus gordonii*, *Streptococcus intermedius*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus parasanguinis*, *Streptococcus pyogenes*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Streptococcus sobrinus* and *Streptococcus vestibularis*.

RESULTS AND DISCUSSION

All serotyping results for types or groups by the Denka kit were completely identical to those obtained from serotyping by using the conventional Quellung test (Table 3). The predominant serotypes were 19, 23, 3, 6, 15, 18 and 11. There was excellent correlation of the results. Sensitivity and specificity were 100 and 100 %, respectively.

The results of serotyping for specific types by the Denka kit were identical to those obtained by the Quellung test. Some serogroups, e.g. 3, 4 and 14, do not have specific types. Specific types were determined for 191 pneumococcal strains and there was excellent correlation of the results. The Denka kit cannot distinguish between specific types 9A and 9V or 24B and 24F. In this study, one pneumococcal strain was identified as 9V by the Quellung test and as 9A or 9V by the Denka kit. Two strains that were identified as 24B by the Quellung test were identified by the Denka kit as either 24B or

Table 1. Composition of the Denka kit: pool sera and type or group sera contained

Pool serum	Type or group sera									
	Vaccine-related					Non-vaccine-related				
1	1	2	3	4	5					
2	6	8	9	10						
3	11	12	14	15		16				
4	17	18	22			21				
5	20	33				29	31	34	35	47
6	23					25	28	41	46	
7						27	32	36	38	39
8	7	19				24	40			
						37*				

*37 is not included in any of the eight pool sera.

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Table 2. Composition of the Denka kit: specific type factor sera and pattern of reaction to factor sera

Specific type	Reaction to factor serum			
Type 6	6b	6c		
6A	+	–		
6B	–	+		
Type 7	7b	7c	7e	7f
7A	+	+	–	–
7B	–	–	+	–
7C	–	–	–	+
7F	+	–	–	–
Type 9	9d	9e	9f	
9A	+	–	–	
9V	+	–	–	
9N	–	+	–	
9L	–	–	+	
Type 10	10b	10c		
10A	–	+		
10F	+	–		
Type 11	11c	11f	11g	
11A	+	–	–	
11B	–	+	+	
11C	+	+	–	
11F	–	–	+	
Type 15	15b	15e	15f	15g
15A	–	–	–	+
15B	+	+	–	–
15C	–	+	–	–
15F	+	–	+	–
Type 18	18c	18d	18e	18f
18A	–	+	–	–
18B	–	–	+	–
18C	+	–	+	–
18F	+	–	–	+
Type 19	19b	19d	19e	19f
19A	–	+	–	–
19B	–	–	+	–
19C	–	–	–	+
19F	+	+	–	–
Type 22	22b	22c		
22A	–	+		
22F	+	–		
Type 23	23b	23c	23d	
23A	–	+	–	
23B	+	–	+	
23F	+	–	–	
Type 24	24b	24c		
24A	–	+		
24B	+	–		
24F	+	–		
Type 32	32b			
32A	+			
32F	–			
Type 33	20b	33b	33e	33f
33A	+	–	–	–
33B	–	–	+	+
33C	–	–	+	–
33F	–	+	–	–
Type 35	20b	29b	35b	42a
35A	+	–	–	–
35B	–	+	–	–
35C	+	–	–	+
35F	–	–	+	–

24F (Table 4). In this study, a Quellung result of 9V or 24B was determined to be identical to the Denka result of 9V/9A or 24B/24F, respectively.

There was no agglutination with the Denka kit against all 18 type strains of streptococci other than pneumococci. All tested negative with all polyvalent pool sera 1–8; hence, there was no need for further serotyping.

Since the 1940s, the *Streptococcus* Unit at Statens Serum Institute, Denmark, has manufactured diagnostic pneumococcal antisera, intended for identification and typing of pneumococci by the capsular reaction test. This method has proved to be reliable and has been used extensively by medical and research centres as well as vaccine industries worldwide. However, the procedure of serotyping is labour-intensive, time-consuming, requires experience and technical skill for interpretation of results and the reagents are not easily affordable, especially by developing countries. Our results show that the Denka kit has excellent correlation with the Quellung test and has several advantages: it is more rapid (mean test time, 5 min, vs 15 min by Quellung test) and easy to perform, requires no special equipment and is much cheaper (0·5 US$ per test, vs 1·4 US$ per Quellung test).

In the past, other serotyping techniques have been described, including the counterimmunoelectrophoresis (CIEP) (Ayyagari *et al.*, 1984), latex agglutination (LA) and coagglutination (COA) methods (Kronvall, 1973; Kaldor *et al.*, 1988; Lalitha *et al.*, 1996). CIEP has the drawback that the polysaccharide capsules of a small number of important serotypes fail to form precipitin lines (Henrichsen *et al.*, 1980). The COA method for serotyping pneumococci was first developed by Kronvall (1973), who reported that it exhibited weak cross-reactions with one or more serotypes in addition to the Quellung serotype. The LA and COA methods rely on elaborate, time-consuming, self-prepared reagents (Kaldor *et al.*, 1988). More recently, a multiplex PCR assay for determination of *S. pneumoniae* serotypes and serogroups has been described (Lawrence *et al.*, 2003). This method can only determine five serotypes, 1, 3, 14, 19F and 23F, and serogroups 6, 19 and 23. It is not yet available for commercial use and would not be easily affordable by developing countries as it is expensive. In contrast, the Denka kit is commercially prepared, cheap and ready for use. The Denka kit offers hope to the majority of developing countries to be able to perform serotyping and obtain the appropriate conjugate vaccine, which will help to prevent high morbidity and mortality from pneumococcal infections in paediatrics (Wafula & Onyango, 1986; Graham, 1990; Garenne *et al.*, 1992; Onyango & Wafula, 1995; Murray & Lopez, 1996). It will also be very useful when conducting large epidemiological surveys. The only limitation is that the Denka kit cannot distinguish between specific types 9A and 9V or 24B and 24F. This kit also does not include some minor non-vaccine types, such as 13, 42, 43, 44, 45 and 48, and some specific types, e.g. 12A, 12B, 12F, 11D, 10B, 10C and 33D. However, this point is not a major problem because the types or groups to which these specific types belong are covered in

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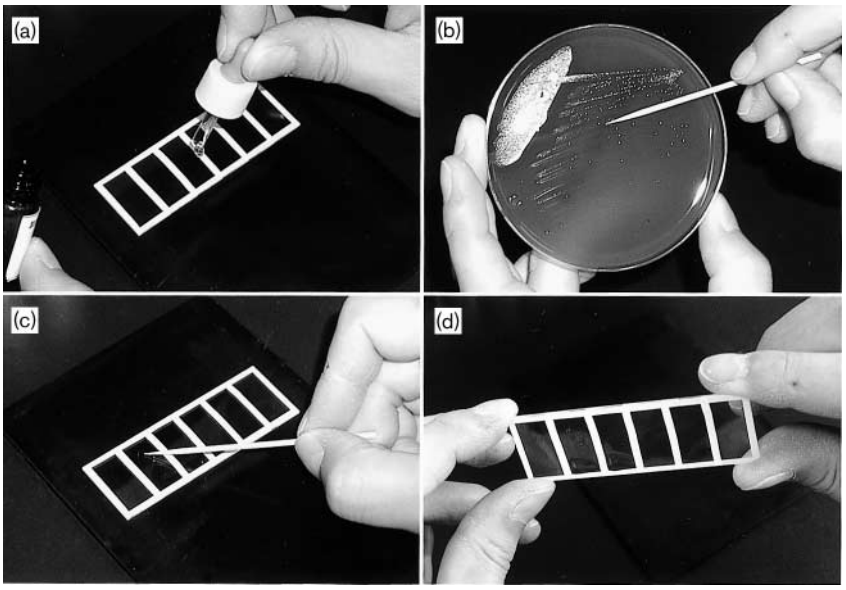

Fig. 1. Procedure of slide-agglutination by using the Denka kit. (a) Drop antiserum onto glass slide; (b) pick colonies of bacteria; (c) mix colonies with antiserum; (d) tilt glass slide back and forth and observe for agglutination.

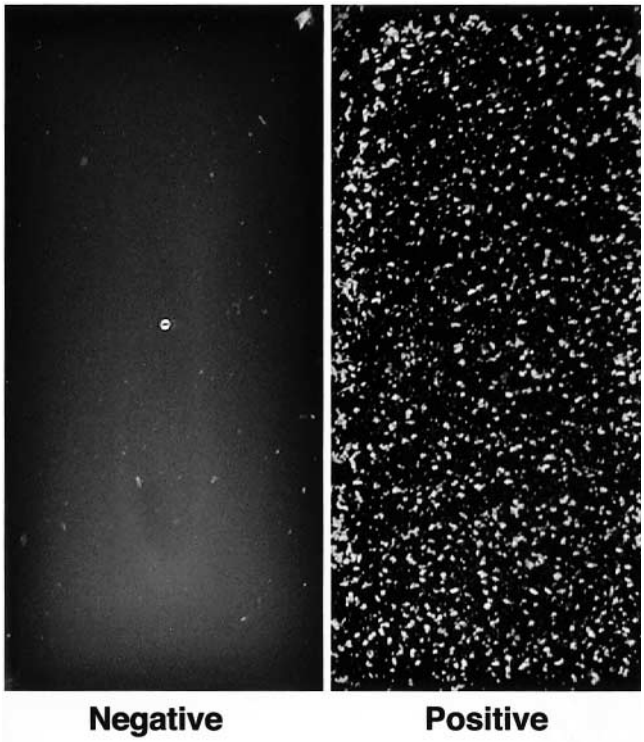

Fig. 2. Results of slide-agglutination by the Denka kit. Left, negative reaction; right, positive reaction.

the kit (Table 1). Serogroups that are not included in the new serotyping kit are minor non-vaccine types that are not important for serotyping.

Our results showed excellent correlation with the conventional method, with sensitivity and specificity of 100 % by

Table 3. Determination of type or group of *S. pneumoniae* strains

Data are no. (%) of isolates serotyped by the Denka kit or Quellung test

Type or group	Denka kit	Quellung test
3	31 (10·9)	31 (10·9)
4	2 (0·7)	2 (0·7)
6	27 (9·5)	27 (9·5)
7	4 (1·4)	4 (1·4)
8	6 (2·1)	6 (2·1)
9	4 (1·4)	4 (1·4)
10	1 (0·4)	1 (0·4)
11	13 (4·6)	13 (4·6)
12	4 (1·4)	4 (1·4)
14	12 (4·2)	12 (4·2)
15	26 (9·1)	26 (9·1)
17	2 (0·7)	2 (0·7)
18	15 (5·3)	15 (5·3)
19	49 (17·2)	49 (17·2)
20	3 (1·1)	3 (1·1)
21	7 (2·5)	7 (2·5)
23	39 (13·7)	39 (13·7)
24	2 (0·7)	2 (0·7)
25	2 (0·7)	2 (0·7)
27	2 (0·7)	2 (0·7)
28	1 (0·4)	1 (0·4)
31	3 (1·1)	3 (1·1)
33	1 (0·4)	1 (0·4)
34	12 (4·2)	12 (4·2)
35	7 (2·5)	7 (2·5)
39	2 (0·7)	2 (0·7)
40	1 (0·4)	1 (0·4)
41	5 (1·8)	5 (1·8)
46	1 (0·4)	1 (0·4)
Total	285 (100)	285 (100)

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Table 4. Determination of specific types of *S. pneumoniae* strains

Data are no. (%) of isolates serotyped by the Denka kit or Quellung test

Specific type	Denka kit	Quellung test
6A	14 (7·4)	14 (7·4)
6B	14 (7·4)	14 (7·4)
7A	1 (0·5)	1 (0·5)
7C	3 (1·6)	3 (1·6)
9A/9V	1 (0·5)	
9V		1 (0·5)
9L	1 (0·5)	1 (0·5)
9N	2 (1·1)	2 (1·1)
10A	1 (0·5)	1 (0·5)
11A	11 (5·8)	11 (5·8)
11B	2 (1·1)	2 (1·1)
11C	2 (1·1)	2 (1·1)
15A	8 (4·2)	8 (4·2)
15B	3 (1·6)	3 (1·6)
15C	9 (4·7)	9 (4·7)
15F	5 (2·6)	5 (2·6)
18A	1 (0·5)	1 (0·5)
18B	2 (1·1)	2 (1·1)
18C	1 (0·5)	1 (0·5)
18F	11 (5·8)	11 (5·8)
19A	4 (2·1)	4 (2·1)
19B	3 (1·6)	3 (1·6)
19F	41 (21·6)	41 (21·6)
23A	6 (3·1)	6 (3·1)
23B	1 (0·5)	1 (0·5)
23F	32 (16·8)	32 (16·8)
24B		2 (1·1)
24B/24F	2 (1·1)	
33F	1 (0·5)	1 (0·5)
35B	1 (0·5)	1 (0·5)
35C	4 (2·1)	4 (2·1)
35F	2 (1·1)	2 (1·1)
Total	191 (100)	191 (100)

using the Denka kit. In conclusion, the Denka kit is easy to use, simple, cheap and reliable. This kit may be very useful, especially for fieldworkers in developing countries who are involved in large epidemiological studies or vaccine development.

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