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Typing of Pneumococci by Using 12 Pooled Antisera

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A new simplified chessboard system for typing of *Streptococcus pneumoniae* is described. It is intended for typing or grouping of 90 to 95% of the pneumococcal strains most commonly isolated from blood or cerebrospinal fluid and is based on 12 pooled diagnostic antisera, each reacting with 7 to 11 single types, together covering the 23 different vaccine-related types as well as 25 other cross-reacting types. Worldwide surveillance of the type distribution is important in order to ensure an optimal formulation of pneumococcal polysaccharide vaccines and, in the future, of polysaccharide-protein conjugate vaccines. The simplified typing system described in this paper makes it easier to carry out surveillance in other than specialized reference laboratories. Finally, it takes advantage of the fact that some types cause disease more often in children—as opposed to adults—than do others.

Pneumococci account for most cases of community-acquired pneumonia (6, 11, 16, 17), and the incidence of pneumococcal meningitis has been estimated to be close to 1.5 cases per 100,000 individuals per year, with an average mortality rate of 30 to 40%. The incidence of pneumococcal bacteremia is around 5 to 10 cases per 100,000 individuals per year, and in adults pneumococcal bacteremia treated with penicillin has a fatality rate of 15 to 24% (2, 3, 5, 6). Because of the unacceptably high mortality rate, despite optimal antibiotic treatment and intensive care, prophylaxis must be offered to those at high risk of death if infected by pneumococci (2, 3, 15). The pneumococcal vaccine currently in use is composed of 23 different capsular polysaccharides representing close to 90% of the types of pneumococci isolated from invasive infections (8, 14, 15, 17). A polysaccharide-protein conjugate vaccine for the prevention of pediatric pneumococcal infections is now being developed.

Surveillance of the distribution of pneumococcal types should be conducted in every country in order to ensure a continued optimal formulation of the existing polysaccharide vaccine and of future protein-conjugated vaccines (6, 9, 14, 15, 17) for the following reasons. (i) Changes occur in the rank order of types isolated from invasive diseases over time (7, 17). (ii) The predominant types differ between patients of different age groups (11). (iii) Regional differences in type distribution have been reported (7, 17), and our knowledge about the type distribution in many parts of the world, especially in the developing countries, is still inadequate (9).

Thus, it is important to clarify whether specially formulated vaccines are needed at different times, for different age groups, for different parts of the world, or for protection against different kinds of pneumococcal infections (7, 9, 17). Another aspect is the increasing number of pneumococcal strains found to be resistant to different antimicrobial drugs. To achieve an optimal surveillance of changes in the pattern of drug resistance, the pneumococci studied should also be typed.

The method traditionally used for typing of pneumococci is the capsular reaction test, the quellung or Neufeld reaction (1, 9, 12). This is the method of choice because it is easy, fast, accurate, and economical (1). The availability of Omniserum (Statens Seruminstitut, Copenhagen, Denmark), a pooled pneumococcal serum that reacts with all types (9, 12, 13), provides clinical microbiology laboratories with an invaluable reagent for rapid identification of pneumococci (1). Most laboratories do not type pneumococcal isolates because of the large number of diagnostic antisera required for typing. A total of 84 different pneumococcal types have been described (4, 12). Types that exhibit close serological cross-reactivity are grouped together. Of the 84 types, 58 belong to 20 groups containing from 2 to 4 types (9, 12) (Table 1), and a total of 46 different pneumococcal types or groups are currently known. Monovalent factor sera rendered specific by multiple absorptions (12) or by induction of immunological tolerance to cross-reacting types previous to immunization (10) for identification of types within groups (9, 12) will not be discussed in this paper.

More than 90% of all pneumococcal strains isolated from blood or cerebrospinal fluid (CSF) belong to one of the 21 different types or groups represented in the 23-valent pneumococcal vaccine (8, 14, 17). A total of 7 pooled sera in addition to 21 type or group sera are needed in order to type or group these strains by the use of the traditional pneumococcal diagnostic antisera. The present paper describes a new, simpler chessboard typing system, based on 12 pooled sera, intended for typing and/or grouping of most pneumococci isolated from blood or CSF.

MATERIALS AND METHODS

Pneumococcal strains. Clinical strains received by the WHO Collaborating Centre for Reference and Research on Pneumococci were examined both by the chessboard typing system and, for control purposes, by the conventional typing system. All strains were optochin sensitive. Pneumococcal type reference strains from the WHO Collaborating Centre were employed for preparation of vaccines for immunization of rabbits and as controls.

Antisera. Conventional pneumococcal diagnostic antisera (9, 12) were from the WHO Collaborating Centre. Five new pools, labelled P to T, of polyvalent antisera were raised in white rabbits (three to five animals per pool) by intravenous injections of formalin-fixed whole-cell vaccines consisting of a mixture of equal parts of 7 to 11 different pneumococcal types (Table 1). The standard procedure for preparation of vaccines and for immunization in use at the WHO Collaborating Centre was followed throughout (12). Blood was drawn from an ear vein of the rabbits once a month.

TABLE 1. A chessboard system for typing and/or grouping of most pneumococci isolated from blood or CSF[a,b]

Existing pool	Type or group with new pool:					Non-vaccine-related type or group
	P	Q	R	S	T	
A	**1**	**18***	**4**	**5**	**2**	
B	**19***	**6***	**3**	**8**		
C	**7***				**20**	24,* 31, 40
D			**9***		**11***	16,* 36, 37
E			**12***	**10***	**33***	21, 39
F				**17***	**22***	27, 32,* 41*
H	**14**	**23***		**15***		13, 28*
G[c]						29, 34, 35,* 42, 47*
I[c]						25, 38, 43, 44, 45, 46, 48

[a] The five pooled sera P to T are composed in such a way that each of the 21 vaccine-related types and/or groups reacts both with one of these sera and with one of the seven pooled sera A to F plus H.

[b] All 46 types or groups are shown in the table (no. 26 and 30 not being in use). Asterisks indicate groups containing the following types: **6**, 6A and **6B**; **7**, **7F**, 7A, 7B, and 7C; **9**, 9A, 9L, **9N**, and **9V**; **10**, 10F and **10A**; **11**, 11F, **11A**, 11B, and 11C; **12**, **12F** and 12A; **15**, 15F, 15A, **15B**, and 15C; 16, 16F and 16A; **17**, **17F** and 17A; **18**, 18F, 18A, 18B, and **18C**; **19**, **19F**, **19A**, 19B, and 19C; **22**, **22F** and 22A; **23**, **23F**, 23A, and 23B; 24, 24F, 24A, and 24B; 28, 28F and 28A; 32, 32F and 32A; **33**, **33F**, 33A, 33B, and 33C; 35, 35F, 35A, 35B, and 35C; 41, 41F and 41A; 47, 47F and 47A. Types and/or groups present in the currently available 23-valent pneumococcal vaccine are indicated by boldface type.

[c] Pools G and I do not react with vaccine types and are, therefore, not included in the chessboard system.

The antisera raised were rendered specific by absorption with small amounts of dense heterologous vaccine suspensions of the cross-reacting types (12). Titers were determined by testing twofold dilutions of the antisera in the capsular reaction test (see below). The titers of the sera obtained from the individual bleedings were determined, and only sera with titers of 8 or higher to all types were mixed and used for further study. The five pools are available from Statens Seruminstitut, as are all the other diagnostic antisera.

Capsular reaction test. Pneumococcal strains were typed by the capsular reaction test (1, 12). The test was carried out by mixing a loopful (a few microliters) of bacterial suspension on a microscope slide with a loopful of antiserum, placing a coverslip on top of the mixture, and subsequently examining the preparation under a microscope, preferably equipped with phase contrast, with an oil immersion lens (magnification, ×100). If the reaction is positive, the capsules become visible because of an in situ immunoprecipitation leading to a change in their refractile index; the bacteria, in addition, agglutinate. The titer of an antiserum was defined as the reciprocal value of the highest dilution still leading to agglutination, but not necessarily to distinctly visible capsules.

Typing and/or grouping of pneumococci. The capsular reaction test is first carried out with, successively, the nine traditional pools (A to I) until a positive reaction is observed. (i) Ordinarily, typing then proceeds by testing the strain in question in antisera against those individual types or groups that are included in the serum pool that gave a positive reaction. (ii) The chessboard method described here instead proceeds by testing for a positive reaction with the new serum pools (P to T). The type or group is then established from the reaction pattern by the use of a table with the types and groups entered in a rectangular chessboard arrangement (Table 1). An example of a typing strategy is shown in Fig. 1.

RESULTS

The types included in the nine pooled pneumococcal typing antisera (A to I) were chosen many years ago so that the most highly cross-reactive types were in the same pool. The types included in the five new pools (P to T) were chosen so that each of the 21 vaccine-related types or groups reacts with both one of these pools and with one of the seven pools A to F plus H (because pools G and I do not contain vaccine types) (Table 1). Sufficiently high titers to all types, without unacceptable levels of interfering cross-reactive antibodies, could be obtained after 2 to 3 months of immunization with the pneumococcal types included in the new pools. Cross-reactions were eliminated by absorption with heterologous vaccines. Finally, the specificities of the five new pooled sera were confirmed by the capsular reaction test with all 84 different type strains. More than 400 pneumococcal strains received by the WHO Reference Centre over a 4-month period were examined by the chessboard system with the conventional typing system as a control. No discrepancies were encountered, either by experienced technicians or by trainees. Some representative examples are given in Table 2. Strain 975 reacted with 2 of the 12 pooled sera used in the chessboard system, and the type of this strain, consequently, could be established directly by the use of Table 1. Strain 981 also reacted with 2 of the 12 pooled sera and was identified as belonging to a group. Isolate 747 was found to react with three different pools, and, therefore, it was believed that this isolate was probably a mixture of two types. This was confirmed by the control test. Strain 719 reacted with one pooled serum only, and it was identified as a capsulated pneumococcal strain of a non-vaccine-related type. Strains 732 and 742 could not be identified because they did not react with any of the 12 pooled sera. The typing results given in the control column of Table 2 were obtained by the successive use of pools A to I and type or group and factor sera.

DISCUSSION

Rearrangement of the types among the pooled sera, A to I, would be inappropriate, because it would only lead to confusion. Therefore, the new chessboard typing system was designed so that seven of the pooled sera could be used without modifications. The two pooled sera G and I were not included in the simplified typing system, because they do not react with any of the vaccine types. The five new pooled sera, P through T, were prepared in such a way that each of the 21 vaccine-related types or groups reacted both with one of these sera and with one of the seven pooled sera, A through F plus H (Table 1). The predominant types were divided among the three pools P to R in such a way that the types responsible for the majority of infections in children are found in pools P and Q, whereas the types most commonly isolated from adults are found in pools P and R (Table 3). Types that are less commonly isolated (Tables 1, 3, and 4) were included in pools S and T in order to ensure the most convenient typing strategy. Of the three vaccine types whose capsular polysaccharides are neutral (i.e., neither negatively nor positively charged), the two common types 7F and 14 are included in pool P, whereas the less frequently isolated type 33F is included in pool T. The percentage of pneumococcal strains isolated from blood or CSF reacting with 1 or 2 of the 12 pooled antisera is given in Table 4, which may also be used for planning of the most optimal typing strategy. Thus, the overall chance of finding a

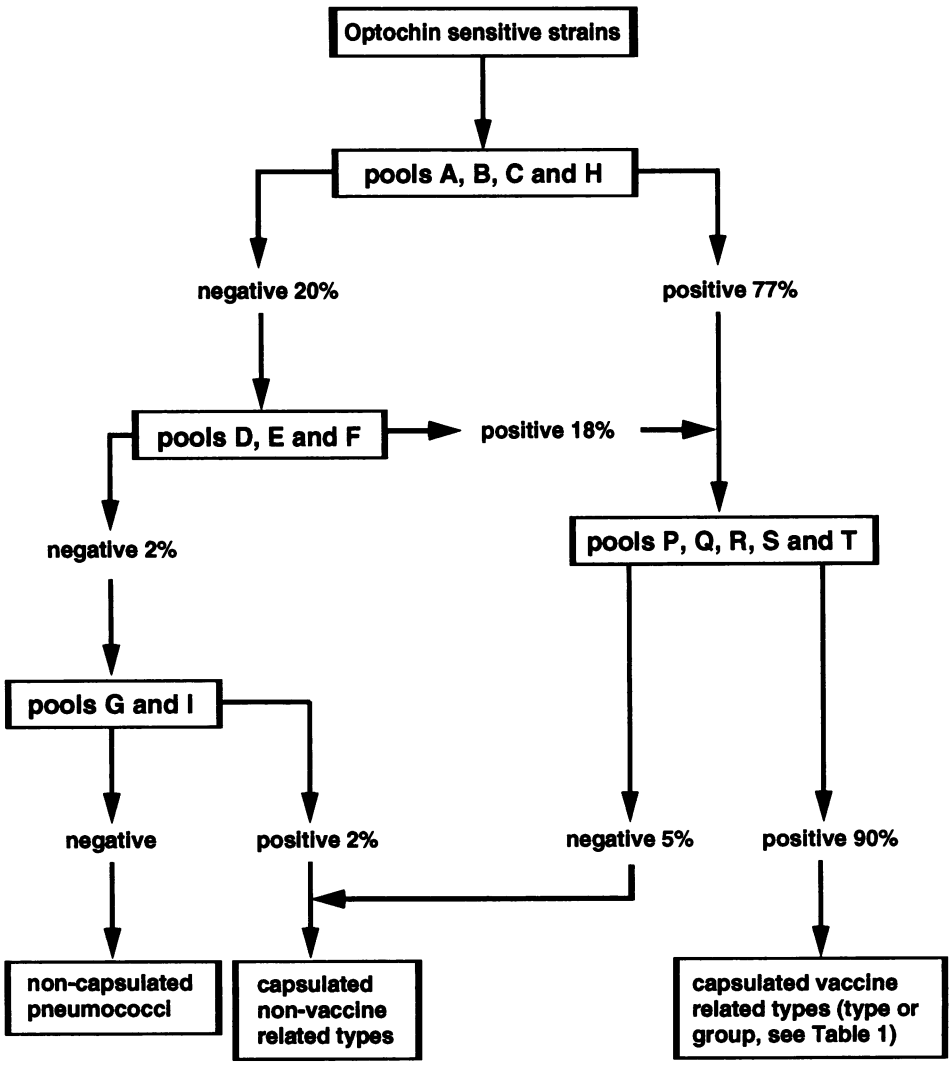

FIG. 1. Flow diagram showing the strategy for typing of pneumococci. More than 90% of strains isolated from either blood or CSF can be typed and/or grouped by the use of 12 pooled sera (pools A to F plus H and pools P to T) in a chessboard system. In addition, 5% (7% if pools G and I are included in the tests) of the strains can be identified as capsulated pneumococci of non-vaccine-related types. When a pneumococcal strain is found to give a positive reaction with more than one pooled serum, the type of the strain can be established by the use of Table 1. Percentages are calculated on the basis of figures given by Nielsen and Henrichsen (14).

positive capsular reaction is around 77% if an unknown pneumococcal strain (i.e., optochin-sensitive, gram-positive diplococcus) is first examined with pools A, B, C, and H. A proposed scheme for typing of pneumococci is given in Fig. 1.

The advantage of the chessboard method for typing of pneumococci can be summarized as follows. Eight different types that do not belong to a group can be identified directly. From the figures given by Nielsen and Henrichsen (14), it was calculated that these types account for around 35% of the more than 10,000 pneumococcal strains that were typed over a period of 6 years by the WHO Collaborating Centre for Reference and Research on Pneumococci in Copenhagen. It was also calculated that another 60% of the strains could be grouped by the simplified typing system and that an additional 5% of the strains could be identified as capsulated pneumococci. Only around 5% of the strains would not react with any of the 12 pooled sera included in the system. Typing according to the classical pneumococcal typing system

TABLE 2. Representative examples of the use of the chessboard typing system for typing and/or grouping of pneumococci

Strain	Pools[a]	Result[b]	Control result[c]
719	H, —	PN	13[d]
732	—, —	?	42[d]
742	—, —	?	R
747	H, Q + S	23*	23B
		15*	15B
975	H, P	14	14
981	H, S	15*	15C

[a] Strains were examined with 12 pooled sera (A to F plus H and P to T [Table 1]). Letter, positive reaction in pool; —, no reaction.

[b] Results interpreted from the reactions with the 12 pooled sera. Asterisks indicate groups containing different types as shown in Table 1, footnote *b*. PN, the strain is found to be a capsulated pneumococcus, which cannot be typed; ?, the type or group of the strain cannot be determined.

[c] Results obtained by the use of the conventional panel of diagnostic typing antisera. Types within groups were distinguished by the use of factor sera. R, a noncapsulated pneumococcus (rough).

[d] Non-vaccine-related type.

TABLE 3. Percentage of pneumococcal strains isolated from blood or CSF reacting with one of the five pooled sera P to T

Age of isolate source[a]	% of strains reacting with pool[b]:					Acc[c]
	P	Q	R	S	T	
≤14	39	32	12	8	3	94
>14	32	14	26	12	8	92

[a] Children were defined as individuals 14 years of age or less; adults were defined as individuals over 14 years of age.
[b] The five pooled sera, P to T, react with vaccine-related types and/or groups only.
[c] Acc, accumulated percentage calculated on the basis of figures given by Nielsen and Henrichsen (14).

would require 28 different antisera in order to achieve the same degree of differentiation.

The simplified chessboard typing system is a useful alternative to the classical pneumococcal typing system not only for clinical microbiology laboratories but also for specialized reference laboratories, because nearly all pneumococcal strains isolated from blood or CSF can be typed or grouped by the use of only 12 pooled sera and because 21 type or group sera can be replaced by the five new pools.

TABLE 4. Percentage of pneumococcal strains isolated from blood or CSF reacting with one or two of the pooled antisera

Pool[a]	% of strains reacting with pool[b]:					Neg[c]	Acc[d]
	P	Q	R	S	T		
A	8.0	5.0	5.3	2.3	0.6		21.2
B	8.6	9.1	6.7	3.7			28.1
C	7.6				1.5	2.0	11.1
D			6.3		1.8	0.8	8.9
E			3.2	1.7	1.0		5.9
F				1.0	1.7		2.7
H	8.7	5.7		2.1		0.6	17.1
G[e]						1.4	1.4
I[e]						0.5	0.5
Acc[d]	32.9	19.8	21.5	10.8	6.6	5.3	96.9

[a] A to I, pooled sera reacting with all known pneumococcal types.
[b] P to T, new pooled sera reacting with the 21 vaccine-related types and/or groups only.
[c] Neg, percentage of strains reacting with pools A through I, but negative when examined with pools P through T (Table 1).
[d] Acc, accumulated percentage. Types accounting for less than 0.3% of the examined strains are not included in the table. Percentages are calculated on the basis of figures given by Nielsen and Henrichsen (14).
[e] Pools G and I do not react with any vaccine-related type.

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