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Rapid and Reliable Identification of *Streptococcus pneumoniae* Isolates by Pneumolysin-Mediated Agglutination

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A pneumolysin-based agglutination test which allows an easy, rapid, cost-effective, and accurate (100% specific and 95% sensitive) discrimination between pneumococci and other related human and animal pathogenic bacterial strains has been assayed.

Streptococcus pneumoniae infections are a cause of high morbidity and mortality worldwide (17, 19). Following the plate microbiological culture of specimens, the routine presumptive identification of pneumococcal colonies relies primarily on their sensitivity to optochin and secondarily on their bile solubility (12, 18, 19); nevertheless, due to an incorrect implementation of procedures or to difficulties in interpreting results, there are pneumococci which seem to be resistant to either of the above treatments, leading to misinterpretations in their characterization (9, 10, 12, 18). Additionally, the identification of pneumococci is further confirmed by the immunodetection of capsular polysaccharide antigens, mainly by latex agglutination. The use of monospecific antibody preparations enables their serovar typing (11, 19). However, this identification is not reliable enough as, on the one hand, up to 20% of pneumococcal isolates are nonserotypeable (4, 7, 20) and, on the other hand, antisera to pneumococcal capsular polysaccharide antigens and/or C-polysaccharide present cross-reactions with nonpneumococcal microorganisms, such as other α-streptococci (23). In contrast, pneumolysin, a 53-kDa protein, is present in virtually all pneumococcal isolates tested (13).

Pneumolysin is a member of the family of thiol-activated cytolysins. These are antigenically related cholesterol-binding pore-forming toxins produced by species of the *Bacillus*, *Clostridium*, *Listeria*, and *Streptococcus* genera (2). Suilysin, expressed by *Streptococcus suis*, is the closest relative to pneumolysin (22).

Pneumolysin is liberated by autolysis of the pneumococcus. As a toxin, it is a multifunctional virulence factor which, among its biological properties, has been shown to have inhibitory effects on neutrophil chemotaxis, phagocytosis, and the respiratory burst, as well as on lymphocyte function, although it is immunogenic during infection (14).

As pneumolysin is a pneumococcal component, we have explored the suitability of a pneumolysin-based agglutination assay to identify pneumococcal isolates. We hereby present data on the usefulness of this approach, which provides a workable alternative methodology to others currently available.

MATERIALS AND METHODS

Antigen. Recombinant pneumolysin—a pTrc 99A construction—was expressed in *Escherichia coli* JM105 and purified from cell lysates by fast protein liquid chromatography with a HiLoad 16/10 phenyl-Sepharose hydrophobic column (Pharmacia), essentially as described elsewhere (16).

Rabbit polyclonal antiserum production. Purified pneumolysin was suspended in 10 mM phosphate-buffered saline, pH 7.3, and emulsified 1:1 in incomplete Freund's adjuvant (Sigma). A New Zealand rabbit was intramuscularly injected at 2-week intervals with 1 ml of immunogen emulsion (5). Immunizations comprised 25 μg, 50 μg, 100 μg (two doses), and 1 mg (eight doses) of purified pneumolysin. Two weeks after the last immunization, the animal was anesthetized and subjected to exsanguination by cardiac puncture (6).

The immunoglobulin G (IgG) fraction of the antiserum was purified after ammonium sulfate precipitation followed by affinity chromatography with protein A-Sepharose (Pharmacia) (3).

Preparation of sensitized staphylococci. Two-milliliter aliquots of this IgG were extensively absorbed with sonicates of *E. coli* JM105 and applied to 1 ml of 10% (vol/vol) suspensions of activated *Staphylococcus aureus* Cowan 1 (ATCC 12598) as described by Kronwall (15). Staphylococci sensitized with normal rabbit IgG reagent-grade fractions (Sigma), absorbed as described above, were similarly prepared to be used as negative controls. To make agglutination more evident, 3 ml of sensitized staphylococcal suspensions was counterstained with 900 μl of the basic blue solution, Colorante 2 (Tinción rápida Grifols; Diagnostic Grifols, S.A., Parets del Vallès, Barcelona, Spain).

Bacterial specimens. The present study included (i) a few reference strains of the family *Enterobacteriaceae*; (ii) nonpneumococcal lysates from reference bacterial strains producing pneumolysin-related toxins, including six different clinical isolates of *Clostridium perfringens*; (iii) beta-hemolytic streptococci (*Streptococcus pyogenes*, *Streptococcus equi* subsp. *equi*, and *Streptococcus equisimilis*); (iv) reference viridans streptococci (21) (*Streptococcus anginosus*, *Streptococcus bovis*, *Streptococcus mitis*, *Streptococcus salivarius*, and *Streptococcus sanguis*) and 50 unselected clinical isolates of this group (47 were recovered from sputa, 2 were recovered from musculoskeletal wounds, and 1 was recovered from a blood culture); (v) several other streptococci; (vi) 22 reference pneumococcal strains belonging to different serotypes; (vii) 10 nontypeable pneumococcal isolates from the Laboratorio de Referencia de Neumococos, Centro Nacional de Microbiología, Madrid, Spain (they were classified as pneumococci based on their sensitivity to optochin and bile solubility); (viii) 20 atypical pneumococcal isolates also received from the Laboratorio de Referencia de Neumococos (these were resistant to optochin and/or insoluble in deoxycholate but positive by hybridization with the specific DNA probe pCE3 [8, 9]); and (ix) 22 pneumococcal isolates recovered from 6-year-old children considered to be healthy carriers (their classification as pneumococci was based on their sensitivity to optochin and bile solubility as described above). The hemolytic activity of lysates from thiol-activated cytolysin-producing strains was also assayed (24).

Bacterial isolates under investigation were grown as lawns on blood agar plates at 37°C for 24 h. Thereafter, bacterial suspensions were prepared in phosphate-buffered saline containing 0.05% Triton X-100, pH 7.3, and spectrophotometrically standardized to an optical density of 2 at 625 nm. These bacterial suspensions were then incubated at 37°C for 30 min. This is a gentle treatment which lyses pneumococci and allows the release of pneumolysin. Lysates-suspensions were afterwards spun down at 15,000 × *g* for 5 min in a bench top Eppendorf 5415 C centrifuge.

Agglutination tests. The assays were carried out essentially as described previously (25), with minor adaptations. Agglutination tests were performed on a smooth white glazed surface. Twenty-five microliters of the final bacterial supernatants was mixed with 25 μl of sensitized staphylococci, covering an elliptical area (approximately 2 by 3.5 cm) with the help of a disposable stick. The mixture was then rocked back and forth by hand. Results were clearly apparent with the naked eye under the regular fluorescent light of the laboratory. A test was considered positive when agglutination occurred within 3 min of mixing the staphylococcal suspensions and the test samples.

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TABLE 1. Agglutination results with antipneumolysin-adsorbed staphylococci

Bacterial strain	Result[a]
Escherichia coli ATCC 25922	−
Serratia marcescens ATCC 8100	−
Salmonella enteritidis PT6a	−
Enterobacter cloacae ATCC 23355	−
Proteus vulgaris ATCC 13315	−
Listeria monocytogenes AP2-415	−
Listeria monocytogenes V12-310	−
Listeria monocytogenes serogroup 1/2a CECT 932	−
Listeria monocytogenes serogroup 1/2b CECT 936	−
Listeria monocytogenes serogroup 4b	−
Listeria monocytogenes Ohio serogroup 4b	−
Listeria monocytogenes Scott A serogroup 4b	−
Listeria monocytogenes serogroup 4b CECT 935	−
Listeria ivanovii serogroup 5 CECT 913	−
Listeria innocua ATCC 33090	−
6 different clinical isolates of *Clostridium perfringens*	−
Streptococcus suis NCTC 10234	−
Streptococcus pyogenes ATCC 19615	−
6 different clinical isolates of *Streptococcus pyogenes*	−
Streptococcus equi subsp. *equi* NCTC 9682	−
Streptococcus equisimilis ATCC 9542	−
Streptococcus anginosus ATCC 12395	−
Streptococcus bovis NCTC 8177	−
Streptococcus mitis ATCC 33399	−
Streptococcus salivarius subsp. *salivarius* ATCC 7073	−
Streptococcus salivarius subsp. *thermophilus* NCTC 10353	−
Streptococcus sanguis ATCC 10556	−
50 unselected clinical isolates of the viridans group	−[b]
Streptococcus equinus ATCC 9812	−
Streptococcus ferus ATCC 33477	−
Streptococcus iniae ATCC 29178	−
Streptococcus oralis ATCC 35037	−
Streptococcus sobrinus ATCC 19435	−
Streptococcus uberis ATCC 19436	−
Streptococcus pneumoniae EF 2226	+
Streptococcus pneumoniae EF 2541	+
Streptococcus pneumoniae EF 8435	+
Streptococcus pneumoniae CCUG 2839A	+
Streptococcus pneumoniae CCUG 1086B	+
Streptococcus pneumoniae 993 ATCC 33400 type 1	−[c]
Streptococcus pneumoniae NCTC 7466 type 2	+
Streptococcus pneumoniae GB05 type 3	+
Streptococcus pneumoniae CCUG 3506 type 7A	+
Streptococcus pneumoniae CCUG 8440 type 11B	+
Streptococcus pneumoniae CCUG 8444 type 12A	+
Streptococcus pneumoniae CCUG 1086B type 14	+
Streptococcus pneumoniae 19C 408/41	+
Streptococcus pneumoniae 11D 70/86DK	+
Streptococcus pneumoniae 12B Gambia 1/81	+
Streptococcus pneumoniae 18C 4593/40	+
Streptococcus pneumoniae 23F Mac	+
Streptococcus pneumoniae 33F 3077/37	+
Streptococcus pneumoniae 10B 10F+A Sri Lanka NR 105/83	+
Streptococcus pneumoniae 17F L Rose	+
Streptococcus pneumoniae 19A 1773/39	+
Streptococcus pneumoniae 20L	+
10 different nontypeable pneumococcal isolates	+
20 atypical pneumococcal isolates	+[d]
22 different pneumococcal isolates from healthy carriers	+

[a] A positive reaction (+) means that agglutination was visible within 3 min of mixing the test sample with the staphylococci.

[b] A background microagglutination was usually developed with both the negative control and the antipneumolysin-sensitized staphylococci.

[c] The lysate assayed was partially hemolytic.

[d] Three lysates were agglutination negative and nonhemolytic.

RESULTS AND DISCUSSION

Agglutination results are shown in Table 1. Lysates from the unselected clinical isolates of the viridans group usually presented a slow-developing weak agglutination which was also visible with the negative control staphylococci; these phenomena were never observed with the other strains tested. Under the assayed conditions, only pneumococcal isolates produced positive results with the antipneumolysin-sensitized staphylococci. This agglutination was generally strong and readable in 90 s. Lysates from *S. pneumoniae* 993 ATCC 33400 type 1 and from three atypical pneumococcal isolates did not induce agglutination; the lysate from the reference strain was partially hemolytic, whereas the other three were not hemolytic at all. When lysates from highly concentrated bacterial suspensions of these isolates were retested, agglutination results remained negative. Supposed producers of listeriolysin O (*Listeria monocytogenes*), ivanolysin (*Listeria ivanovii*), perfringolysin O (*C. perfringens*), suilysin (*S. suis*), or streptolysin O (*S. pyogenes*) did not elicit agglutination. What is more, amounts as low as 5 ng of purified pneumolysin induced agglutination, whereas 1 μg of commercially available streptolysin O or alpha-hemolysin from *S. aureus* (Sigma), or purified alpha-hemolysin from *E. coli*, did not.

The absorbed pneumolysin-specific rabbit IgG preparation developed an intense single band of 53 kDa when assayed by Western blotting against 500 ng of purified pneumolysin but a barely visible one against streptolysin O. Five randomly selected pneumococcal lysates showed a band smear with a major band of 53 kDa; a similar pattern of a few fainter bands was visible when lysates from three strains of *L. monocytogenes*, one of *L. ivanovii*, three of *C. perfringens*, one of *S. suis*, and three of *S. pyogenes* were tested. The above-mentioned agglutination-negative lysates presented only faint bands when the Western blot was developed by using an enhanced chemiluminescence substrate (peroxidase; Boehringer Mannheim).

Our observations show that this pneumolysin-based test is (i) an easy, cost-effective, and rapid method for the identification of pneumococcal isolates (sample preparation does not take more than 45 min, nor does it require any additional routine laboratory equipment; agglutination results are read in less than 3 min); (ii) 100% specific, in spite of the observed cross-reactions between the antipneumolysin polyclonal serum that we used and the antigenically related toxins; and (iii) 95% sensitive. Pneumococcal strains seem to produce variable amounts of pneumolysin, as judged by their hemolytic activities (1, 13); a detailed analysis of the extent of pneumolysin production by pneumococcal isolates is lacking. Those agglutination-negative lysates seem to correspond to low-pneumolysin-producing isolates as hemolysis and enhanced chemiluminescence Western blot analyses have shown. Overall, we conclude that this pneumolysin-mediated agglutination enables an easy and accurate discrimination between pneumococci and other human and animal pathogenic bacterial strains and is therefore of high microbiological diagnostic value; in an improved format, its sensitivity could be even higher than that reported in this paper and could also be applied to the detection of pneumolysin in biological samples in order to speed up the diagnosis of pneumococcal infections.

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REFERENCES

1. **Alexander, J. E., R. A. Lock, C. C. A. M. Peeters, J. T. Poolman, P. W. Andrew, T. J. Mitchell, D. Hansman, and J. C. Paton.** 1994. Immunization of

mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. Infect. Immun. **62:**5683–5688.
2. **Alouf, J. E., and C. Geoffroy.** 1991. The family of the antigenically-related cholesterol-binding ('sulphydryl-activated') cytolytic toxins, p. 147–186. *In* J. E. Alouf and J. H. Freer (ed.), Sourcebook of bacterial protein toxins. Academic Press, London, United Kingdom.
3. **Andrew, S. M., and J. A. Titus.** 1997. Purification and fragmentation of antibodies, p. 2.7.1–2.7.6. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), Current protocols in immunology. John Wiley and Sons, New York, N.Y.
4. **Cima, M. D., B. López, F. Vázquez, F. Pérez, A. Fleites, and F. J. Méndez.** 1996. Epidemiological study of *Streptococcus pneumoniae* strains from hospitalized carriers, p. 93–96. *In* 10th Mediterranean Congress of Chemotherapy. Monduzzi Editore, Bologna, Italy.
5. **Cooper, H. M., and Y. Paterson.** 1995. Production of antibodies, p. 2.4.1–2.4.9. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), Current protocols in immunology. John Wiley and Sons, New York, N.Y.
6. **Donovan, J., and P. Brown.** 1995. Euthanasia, p. 1.8.1–1.8.4. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), Current protocols in immunology. John Wiley and Sons, New York, N.Y.
7. **Fenoll, A., I. Jado, D. Vicioso, and J. Casal.** 1997. Dot blot assay for the serotyping of pneumococci. J. Clin. Microbiol. **35:**764–766.
8. **Fenoll, A., I. Jado, D. Vicioso, A. Pérez, and J. Casal.** 1998. Evolution of *Streptococcus pneumoniae* serotypes and antibiotic resistance in Spain: update (1990 to 1996). J. Clin. Microbiol. **36:**3447–3454.
9. **Fenoll, A., J. V. Martínez-Suárez, R. Muñoz, J. Casal, and J. L. García.** 1990. Identification of atypical strains of *Streptococcus pneumoniae* by a specific DNA probe. Eur. J. Clin. Microbiol. Infect. Dis. **9:**396–401.
10. **Gardam, M. A., and M. A. Miller.** 1998. Optochin revisited: defining the optimal type of blood agar for presumptive identification of *Streptococcus pneumoniae*. J. Clin. Microbiol. **36:**833–834.
11. **Henrichsen, J.** 1995. Six newly recognized types of *Streptococcus pneumoniae*. J. Clin. Microbiol. **33:**2759–2762.
12. **Isenberg, H. D. (ed.).** 1995. Clinical microbiology procedures handbook, p. 1.20.19–1.20.20 and 1.20.25–1.20.26. ASM Press, Washington, D.C.
13. **Kanclersky, K., and R. Möllby.** 1987. Production and purification of *Streptococcus pneumoniae* hemolysin (pneumolysin). J. Clin. Microbiol. **25:**222–225.
14. **Kilian, M.** 1998. *Streptococcus* and *Lactobacillus*, chapter 28 (CD-ROM). *In* A. Balows and B. I. Duerden (ed.), Topley & Wilson's microbiology and microbial infections, 9th ed., vol. 2. Systematic bacteriology. Arnold Publishers, London, United Kingdom.
15. **Kronwall, G.** 1973. A rapid slide-agglutination method for typing pneumococci by means of specific antibody adsorbed to protein A-containing staphylococci. J. Med. Microbiol. **6:**187–190.
16. **Mitchell, T. J., J. A. Walker, F. K. Saunders, P. W. Andrew, and G. J. Boulnois.** 1989. Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties. Biochim. Biophys. Acta **1007:**67–72.
17. **Mitchell, T. J., and P. W. Andrew.** 1995. Vaccines against *Streptococcus pneumoniae*, p. 93–117. *In* D. A. A. Ala'Aldeen and C. E. Hormaeche (ed.), Molecular and clinical aspects of bacterial vaccine development. John Wiley & Sons, New York, N.Y.
18. **Mundy, L. S., E. N. Janoff, K. E. Schwebke, C. J. Shanholtzer, and K. E. Willard.** 1998. Ambiguity in the identification of *Streptococcus pneumoniae*. Optochin, bile solubility, quellung, and the AccuProbe DNA probe tests. Am. J. Clin. Pathol. **109:**55–61.
19. **Musher, D. M.** 1995. *Streptococcus pneumoniae*, p. 1811–1826. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Mandell, Douglas and Bennett's principles and practice of infectious diseases, 4th ed. Churchill Livingstone, New York, N.Y.
20. **Ridgway, E. J., C. H. Tremlett, and K. D. Allen.** 1995. Capsular serotypes and antibiotic sensitivity of *Streptococcus pneumoniae* isolated from primary-school children. J. Infect. **30:**245–251.
21. **Ruoff, K. L.** 1995. *Streptococcus*, p. 299–307. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. ASM Press, Washington, D.C.
22. **Segers, R. P. A. M., T. Kenter, L. A. M. de Haan, and A. A. C. Jacobs.** 1998. Characterisation of the gene encoding suilysin from *Streptococcus suis* and expression in field strains. FEMS Microbiol. Lett. **167:**255–261.
23. **Sørensen, U. B. S.** 1994. Pneumococcal polysaccharide antigens: capsules and C-polysaccharide. An immunochemical study. Lægeforeningens Forlag, Copenhagen, Denmark.
24. **Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois.** 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect. Immun. **55:**1184–1189.
25. **Zollinger, W. D., and J. Boslego.** 1997. Immunologic methods for diagnosis of infections by gram-negative cocci, p. 473–483. *In* N. R. Rose, E. Conway de Macario, J. D. Folds, H. C. Lane, and R. M. Nakamura (ed.), Manual of clinical laboratory immunology, 4th ed. ASM Press, Washington, D.C.