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Cross-Reactions Between α-Streptococci and Omniserum, a Polyvalent Pneumococcal Serum, Demonstrated by Direct Immunofluorescence, Immunoelectroosmophoresis, and Latex Agglutination

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In recent years several groups have used serological methods to demonstrate pneumococcal capsular antigens in sputum. In the present study 123 strains of alpha-hemolytic streptococci (including 97 strains from sputum or pharyngeal specimens) were tested for cross-reactions with a polyvalent antipneumococcal serum (Omniserum). Representatives of the following species were included: *Streptococcus bovis*, *S. equinus*, *S. intermedius*, *S. lactis*, *S. milleri*, *S. mitis*, *S. mutans*, *S. sobrinus*, *S. salivarius*, *S. sanguis*, *S. suis*, and *Aerococcus viridans*. Serological reactions were detected by direct immunofluorescence, immunoelectroosmophoresis, and latex agglutination. Fifteen (12%) of the strains gave positive reactions by all three methods. Positive reactions were also observed with another 32 strains (26%) with two of the methods, whereas 37 strains (30%) gave positive reactions by just one technique. Altogether 84 (68%) strains gave positive reactions with one or more of the methods. Latex agglutination gave positive reactions with 26 (21%) strains compared with 57 (46%) in immunofluorescence and 63 (51%) in immunoelectroosmophoresis. Absorption of the antiserum with one alpha-hemolytic strain reduced but did not entirely eliminate the cross-reactions with five tested strains. These findings indicate a potential risk of cross-reactions with polyvalent antipneumococcal serum in tests carried out on sputa or other specimens which may be contaminated with alpha-hemolytic streptococci.

Streptococcus pneumoniae is a well-recognized etiological agent in respiratory tract infections. It can be isolated by standard bacteriological procedures from pharyngeal, sputum, and blood specimens of patients with pneumonia. In the last 10 years many groups have used serological methods to demonstrate pneumococcal capsular antigens in blood, urine, or sputum to decrease the time for diagnosis and to increase the diagnostic sensitivity. Immunoelectroosmophoresis (IEOP) (3, 10, 13, 16, 17), coagglutination (5, 9), and latex agglutination (LA) (2) have been used, and positive results with these methods have sometimes been claimed to indicate a pneumococcal etiology in spite of negative cultures. The sera used in these investigations were a polyvalent antiserum (Omniserum; State Serum Institute, Copenhagen, Denmark) containing antibodies against the 83 known types of pneumococci, 9 pooled sera, and 46 type sera (12). These antisera are produced for the typing of pneumococci by the capsular reaction test (Neufeld reaction), but IEOP can also be applied (8). However, these antisera have been used for antigen detection with IEOP and coagglutination in sputum which may be contaminated with organisms from the upper respiratory tract.

In our efforts to improve the laboratory diagnosis of pneumococcal pneumonia we have applied several methods, including direct immunofluorescence (IF), to demonstrate pneumococci in clinical specimens by using Omniserum. In preliminary specificity tests we found this antiserum to react with some strains of α-streptococci. These studies were therefore extended to include a collection of α-streptococci which were tested with Omniserum with the use of direct IF, IEOP, and LA.

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MATERIALS AND METHODS

Bacterial strains. In all, 123 alpha-hemolytic streptococci were tested. Of these strains 97 were isolated from sputum or from pharyngeal specimens from patients with symptoms of respiratory tract infections. They were collected at the Örebro Medical Center Hospital, Örebro, Sweden. The study was extended with 13 stock strains from the Dental School, The London Hospital Medical College, London, U.K., and another 13 strains were kindly supplied by E. Falsen, University of Gothenburg, Gothenburg, Sweden. The strains of alpha-hemolytic streptococci were identified by using the short set of biochemical tests described by Hardie and Bowden (7). These tests consist of the fermentation of mannitol and sorbitol, hydrolysis of esculin, production of ammonia from arginine, acetylmethyl carbinol from glucose, and extracellular polysaccharide from sucrose, and hydrogen peroxide formation on plates (detected with Merckoquant Peroxid-Test strips; E. Merck AG, Darmstadt, Federal Republic of Germany). An unencapsulated *S. pneumoniae* strain, CCUG 2497, and three pneumococcal strains belonging to serotypes 4, 6B, and 19F, isolated from clinical specimens of patients with pneumococcal disease, were used as controls. Serotyping of the latter pneumococci was carried out through the courtesy of J. Henrichsen, State Serum Institute.

IF. Omniserum, a polyvalent antipneumococcal antiserum against 83 pneumococcal serotypes, was purchased from the State Serum Institute. The globulin portion was prepared and conjugated with fluorescein isothiocyanate (4). Fluorescein/protein ratios of the conjugates varied from 8.5

$\times 10^{-3}$ to 11.5×10^{-3}. They were used at a dilution of 1:5 to stain smears of pneumococci and α-streptococci which were prepared from cultures grown overnight in broth supplemented with 10% horse serum. The organisms were sedimented by centrifugation and resuspended in saline (phosphate-buffered saline). A small amount of this suspension, containing approximately 1.5×10^6 to 2×10^6 bacteria, was placed on a circular area (about 1 cm^2) on glass slides, dried in air, and gently fixed by heat. One drop of the conjugate was then added. The slides were kept at 37°C in a moist chamber for 30 min, washed twice with phosphate-buffered saline, and mounted with a cover glass. $F(ab')_2$ fragments were prepared from the conjugants by digestion with pepsin (6) and used to stain α-streptococci and pneumococci for true immune reactions. The efficiency of digestion was checked with the use of protein A-containing staphylococci (Cowan I strains) by staining and coagglutination tests. The staphylococci were stained by the Omniserum conjugate before but not after pepsin digestion. Protein A-containing staphylococci, sensitized with the Omniserum conjugate, coagglutinated known pneumococcal serotypes. Corresponding tests with the pepsin-digested conjugate were negative.

The Omniserum conjugate was absorbed with α-streptococci by mixing 1 part of the conjugate with 1 part of sedimented bacteria. The mixture was incubated at 37°C for 4 h and washed twice. Microscopy was carried out with a Zeiss Standard 14 fluorescence microscope with incident light, using narrow blue band activation and LP 520 as a secondary filter. The lamp was an Osram HBO 50 mercury lamp and the slides were read at a magnification of ×420. The criteria for recording the specific staining of bacteria were as follows: 3+ = bright or very bright peripheral staining; 2+ = faint but clearly visible peripheral staining; 1+ = barely visible peripheral staining; − = no reaction. 2+ and 3+ reactions were considered positive.

Agar gel electrophoresis. IEOP was carried out with the use of Rapidophor equipment (Immuno-AG, Vienna, Austria). The thickness of the agarose gel was 1 mm, and a barbital buffer of pH 8.2 was used. Omniserum was used as antibody, and the sediment of α-streptococci and pneumococci grown overnight in dextrose broth or broth supplemented with 10% serum was used as antigen. Approximately 0.10 ml of the reactants was used to fill the wells; these were 4 mm in diameter, separated by a distance of 4 mm from the edges. The uninoculated broth used for growing the bacteria was used as control antigen.

Electrophoresis was run at room temperature for 30 min at a voltage of 13 V/cm, after which the agarose slides were read in oblique light under a magnifying glass. The criteria used for recording the results were as follows: 3+ = strong and broad precipitin line; 2+ = thin and clearly visible precipitin line; 1+ = barely visible precipitin line; − = no visible precipitin line. 2+ and 3+ reactions were considered positive.

In addition, a few tests were carried out with 2-mm-thick standard agarose gel slides (1% agarose [Miles] in barbital buffer, pH 8.6; ionic strength, 0.025) run in a cooled electrophoresis vessel (LKB, Bromma, Sweden) for 60 min with a potential gradient of 6 V/cm and 0.067 ionic strength barbital buffer, pH 8.6. After electrophoresis the plates were washed, dried, and stained with Coomassie blue (1).

LA. The latex slide agglutination test was performed according to the directions of the manufacturer (BBL Pneumoslide TM test for *S. pneumoniae*; BBL Microbiology Systems, Cockeysville, Md.). The α-streptococci and pneumococci were cultured on blood agar plates incubated in candle jars for 18 to 24 h. The size of the colonies varied among the different α-streptococci and therefore 1 to 20 colonies, mostly 2 to 5, were selected and carefully emulsified in 50 μl of saline on a glass slide to obtain a slightly opalescent suspension. One drop of the latex suspension, coated with Omniserum, was added and the reaction was read after 3 min of rocking. Negative controls were performed for each strain. The criteria used for recording the results were as follows: 3+, strong agglutination with clearing of surrounding fluid; 2+, obvious visible agglutination but no clearing; 1+, barely visible agglutination; −, no reaction. 2+ and 3+ were considered positive.

RESULTS

Of the 97 α-streptococci from clinical samples, 80 strains were shown to be *S. mitis* (also known sometimes as *S. mitior*) and 1 strain was *S. sanguis*. Sixteen strains could not be identified by the methods used. The identity of the 26 stock strains is shown in Table 1.

The results of the IF, IEOP, and LA tests, the majority of which were read by two persons, are summarized and

TABLE 1. α-Streptococcal strains tested for cross-reactions in Omniserum

Strain[a]	No.	Reaction by:		
		IF	IEOP	LA
Clinical samples				
S. mitis	80	38+	48+	22+
S. sanguis	1	1+	1+	1−
Unclassified	16	9+	11+	3+
Stock strains				
S. bovis CCUG 4214 (=NCTC 8133)		−	−	−
S. equinus CCUG 11666 (=LRA 58.04.78)		−	−	−
S. equinus CCUG 4213 (=NCTC 10386)		+	−	−
A. intermedius CCUG 11578		−	−	−
S. lactis CCUG 7980 (=NCTC 6681)		−	−	−
S. milleri DOM H117		−	−	−
S. milleri DOM T269		−	−	−
S. milleri DOM T21		+	−	−
S. milleri CCUG 9569 (=NCTC 10708)		+	+	−
S. milleri I CCUG 11667 (LRA 207.11.78)		−	−	−
S. milleri II CCUG 11668 (LRA 37.02.80)		+	−	−
S. mutans CCUG 15479		−	−	−
S. mutans DOM M475		−	−	−
S. mutans DOM T491		−	−	−
S. sobrinus DOM B542		−	−	−
S. sobrinus DOM B276		−	−	−
S. sobrinus DOM 279		−	−	−
S. salivarius DOM A385		+	−	−
S. salivarius DOM H53		+	−	−
S. salivarius CCUG 2654		+	+	+
S. sanguis DOM PC 3827		+	−	−
S. sanguis DOM P695		−	−	−
S. sanguis DOM M371		+	+	−
S. suis 1 CCUG 7985 (=NCTC 10237)		−	−	−
S. suis 2 CCUG 7984 (=NCTC 10234)		−	−	−
A. viridans CCUG 216 (=NCTC 7596)		−	−	−
		57+	63+	26+
S. pneumoniae rough, CCUG 2497		−	−	−

[a] CCUG, Culture Collection, University of Göteborg, Gothenburg, Sweden; NCTC, National Collection of Type Cultures, London, U.K.; LRA, Laboratoire de Recherche api, Moutalieu-Vercieu, France; DOM, Department of Oral Microbiology, Dental School, The London Hospital Medical College, London, U.K.

compared in Fig. 1. As can be seen, 15 α-streptococcal strains (12%) gave positive reactions with all three methods. Positive reactions were also noted for another 27 strains (22%) by both IF and IEOP, three strains by IF and LA, and two strains by IEOP and LA. An additional 37 strains (30%) gave positive reactions by one of the methods. An unencapsulated pneumococcal strain, CCUG 2497, was negative in all three tests (Table 1).

Five tested α-streptococcal strains and two pneumococcal strains which gave 2+ or 3+ reactions in the Rapidophor system gave positive results with our standard agar gel slides (see Materials and Methods). However, there were qualitative differences in that the reactions with α-streptococci in the Rapidophor system were stronger. Table 2 illustrates the results obtained in direct IF tests with the Omniserum $F(ab')_2$ conjugate and the Omniserum conjugate before and after absorption. The unabsorbed $F(ab')_2$ conjugate gave somewhat weaker but on the whole similar results to the unabsorbed Omniserum conjugate with both pneumococci and α-streptococci, indicating that the staining of the latter was not Fc mediated but due to true immune reactions. Absorption of the Omniserum conjugate with an α-streptococcus strain, T-833, eliminated the cross-reactions with the homologous strain and one other *S. mitis* strain (T-893), but the reaction with pneumococci was left unchanged. Cross-reactions with two other strains of *S. mitis* were also reduced but not completely eliminated by absorption of the conjugates.

DISCUSSION

Cross-reactions between capsular polysaccharides of pneumococci and α-streptococci have been reported earlier. Using capsular swelling, Lund in 1950 (11) described eight strains of nonhemolytic streptococci (six *S. mitis* and two *S. sanguis*) which reacted with serum produced against various pneumococcal types. Yurchah and Austrian (20), using the same test, described cross-reactions in 22 different pneumococcal type sera with 50% of alpha- or nonhemolytic streptococci cultured from the respiratory tract. Sottile and Rytel (15), using IEOP, also described cross-reactions in Omniserum with 24 of 77 (31%) α-streptococci. Fourteen of these 24 strains also gave precipitation reactions with different type sera. Thus, the use of type sera reduced but did not eliminate the problem with cross-reactions.

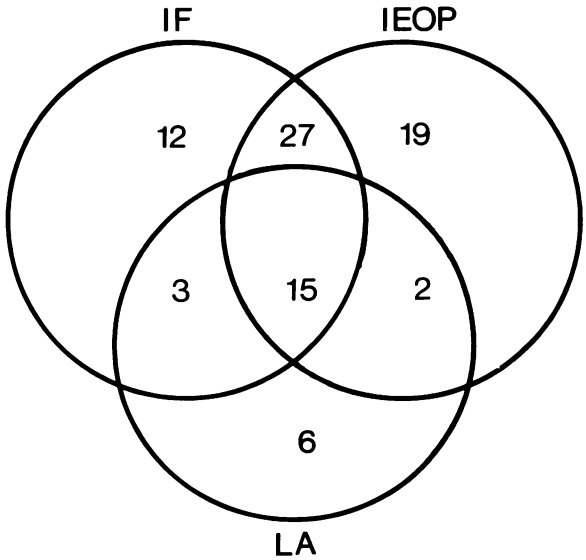

FIG. 1. Cross-reactions with 84 of 123 α-streptococci in Omniserum demonstrated with direct IF, IEOP, and LA.

TABLE 2. Results of direct IF with Omniserum, pepsin-digested Omniserum, and absorbed Omniserum[a]

Strain	Omniserum conjugate	Omniserum $F(ab')_2$ conjugate	Omniserum conjugate absorbed with T-833
S. pneumoniae, type 19F	3+	3+	3+
S. pneumoniae, type 4	3+	3+	3+
S. pneumoniae, type 6B	2–3+	1–2+	2+
S. mitis T-805	3+	2–3+	3+
S. mitis T-833	3+	3+	–
S. mitis T-842	3+	1–2+	1–2+
S. mitis T-844	3+	2+	2–3+
S. mitis T-893	2–3+	2–3+	–
Cowan I	2–3+	–	2+

[a] For grading of reactions, see text.

In the present study, using different methods, 84 of 123 (68%) strains of α-streptococci reacted with Omniserum in one, two, or all three of the methods used, which indicates that pneumococci and α-streptococci have antigens in common. The frequency of detection of cross-reactions varied with the different methods, suggesting either that their sensitivity of detection varies or that different antigenic determinants were detected. The use of several controls each day is helpful of standardization and reproducibility of test results. Nevertheless, the borderline between positive and negative reactions is always to some extent dependent on the subjective estimation of the reader.

With IEOP 63 (51%) α-streptococcal strains produced positive precipitation reactions. Many of these gave thinner and weaker lines than those obtained with the three pneumococcal strains, which all yielded 3+ reactions in the test. However, quite a few of the precipitation reactions from the α-streptococci were strong and distinct and could not be distinguished from those obtained with pneumococci.

By direct IF, 57 of the α-streptococci (46%) gave clearly visible or bright visible peripheral staining. The positive controls always showed 3+ reactions with bright or often very bright peripheral staining. The possibility of Fc-mediated reactions with the α-streptococci in IF was excluded by tests with $F(ab')_2$ conjugates. IF has the additional advantage of allowing a morphological estimation. The α-streptococci were often observed to be smaller and arranged in short chains, and this enabled them to be distinguished from the pneumococci. However, some of the α-streptococci were brightly stained and could not be morphologically differentiated from the pneumococci. In contrast to our results, Wicher et al. (19) obtained cross-reactions with only 4 of 53 (7%) α-streptococci, using Omniserum and indirect IF, and they suggested that with a little experience these 4 strains could also be differentiated from the pneumococci. One possible explanation for the different findings could be the use of indirect IF rather than direct IF. Another source of variation is the selection of the strains of α-streptococcal species used in the two studies. However, since Wicher et al. did not identify their strains to species level, detailed comparisons are not possible.

By the LA method, cross-reactions occurred with 26 strains (21%). This is lower than with either IF or IEOP. It is possible that, unintentionally, there was some bias against

scoring positive results in this test by setting particularly rigorous criteria. With LA we also tested 39 pneumococcal strains which gave 3+ reactions and a few 2+. Four pneumococcal strains did not give positive agglutination when emulsions were made with only 2 to 3 colonies but did so when 10 to 20 colonies were used. These strains all had small colonies, implying that the cell mass used was too small initially. An attempt was therefore made to standardize the bacterial mass, which usually meant using 2 to 5 colonies for most α-streptococci but with a variation of between 1 and 20. All of the tested α-streptococci had negative controls, and no clumping reactions were seen as described by Smith and Washington (14). Most of the positive reactions showed clearly visible agglutination without clearing of surrounding fluid (i.e., 2+ reaction); such reactions were scored as unmistakably positive tests by an experienced technician and one of the authors.

A rather surprising aspect of this investigation was the very restricted range of streptococcal species isolated from the 97 clinical samples. Eighty of these strains (82%) were shown to be *S. mitis*, of which as many as 64 (80%) were positive by one, two, or all three of the methods used. There was a discrepancy between these 97 α-streptococci and the 26 stock strains, the latter giving positive reactions with 9 strains (35%) by IF, 3 strains (12%) by IEOP, and only 1 strain (4%) by LA. Among the stock strains different species were shown to be positive in the tests; three of six *S. milleri*, two of three *S. sanguis*, and three of three *S. salivarius*, one of which was positive by all three methods. Other species such as *S. mutans*, *S. sobrinus*, and *S. suis* were all negative, implying that there are differences between different species. The rough unencapsulated strain of pneumococci did not show any positive reactions with the three method used, indicating that the cross-reacting antigens of the pneumococci are capsular and not somatic in origin.

Absorption of antisera is the standard procedure for elimination of serological cross-reactions. Tugwell and Greenwood (18) were apparently able to eliminate the cross-reactions of *S. viridans* in Omniserum by absorption of the serum with α-streptococci. Although some cross-reactivity was removed by absorption in the present study, the results show that absorption with more than one strain will be necessary if the conjugates are to be used for detection of pneumococci in specimens such as sputum. We would like to point out, however, that this procedure is not necessary for conjugates applied to demonstrate pneumococci in specimens such as cerebrospinal fluid which are usually not contaminated with other bacteria.

In the study reported here, sputum samples have not been routinely tested against Omniserum or type sera in IEOP or coagglutination. Perlino and Schulman (13) showed that saliva from 83 normal individuals failed to give positive tests for Omniserum in IEOP despite the presence of alpha-hemolytic strains on culture. These findings may indicate that a high amount of antigen in the sample is necessary for cross-reactions to be detected. The possibility of cross-reactions with α-streptococci is often not mentioned in discussions about the detection of pneumococcal antigen in sputum by serological methods. Our findings indicate that such cross-reactions constitute an obvious risk in tests of sputa or other specimens which may be contaminated with various α-streptococcal species. If Omniserum is used, the LA test seems most useful because it produces the smallest number of cross-reactions.

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LITERATURE CITED

1. **Axelsen, N. H., J. Krøll, and A. Weelce.** 1973. A manual of quantitative immunoelectrophoresis. Methods and applications. Scand. J. Immunol. **2**(Suppl. 1):26–27.
2. **Browne, K., J. Miegel, and K. D. Stottmeier.** 1984. Detection of pneumococci in blood cultures by latex agglutination. J. Clin. Microbiol. **5**:649–650.
3. **Coonrod, J. D., and M. W. Rytel.** 1973. Detection of type-specific pneumococcal antigens by counter immunoelectrophoresis. J. Lab. Clin. Med. **81**:778–786.
4. **Danielsson, D., and U. Forsum.** 1975. Diagnosis of Neisseria infections by defined immunofluorescence. Methodologic aspects and applications. Ann. N.Y. Acad. Sci. **254**:334–349.
5. **Edward, E. A., and J. D. Coonrod.** 1980. Coagglutination and counterimmunoelectrophoresis for detection of pneumococcal antigens in the sputum of pneumonia patients. J. Clin. Microbiol. **5**:488–491.
6. **Forsum, U.** 1972. Characterization of FITC-labelled $F(ab')_2$ fragments of IgG and a rapid technique for the separation of optimally labelled fragments. J. Immunol. Methods **2**:183–195.
7. **Hardie, J. M., and G. H. Bowden.** 1976. The physiological classification of oral viridans streptococci. J. Dent. Res. **55**:A166–A176.
8. **Henrichsen, J., E. Berntsson, and B. Kaijser.** 1980. Comparison of counterimmunoelectrophoresis and the capsular reaction test for typing of pneumococci. J. Clin. Microbiol. **6**:589–592.
9. **Kalin, M., A. A. Lindberg, and E. H. Olavison.** 1982. Diagnosis of pneumococcal pneumonia by coagglutination and counter immunoelectrophoresis of sputum samples. Eur. J. Clin. Microbiol. **2**:91–96.
10. **Leach, R. P., and J. D. Coonrod.** 1977. Detection of pneumococcal antigens in the sputum in pneumococcal pneumonia. Am. Rev. Respir. Dis. **116**:847–851.
11. **Lund, E.** 1950. Antigenic relationship between pneumococci and non-hemolytic streptococci. Acta Pathol. Microbiol. Scand. **27**:110–118.
12. **Lund, E., and J. Henrichsen.** 1978. Laboratory diagnosis, serology and epidemiology of *Streptococcus pneumoniae*, p. 241–262. *In* T. Bergan and J. R. Norris (ed.), Methods in microbiology, vol. 12. Academic Press, Inc., New York.
13. **Perlino, C. A., and J. A. Schulman.** 1976. Detection of pneumococcal pneumonia by counter immunoelectrophoresis. J. Lab. Clin. Med. **87**:496–502.
14. **Smith, S. K., and J. A. Washington.** 1984. Evaluation of the Pneumoslide latex agglutination test for identification of *Streptococcus pneumoniae*. J. Clin. Microbiol. **20**:592–593.
15. **Sottile, M., and M. W. Rytel.** 1975. Application of counter-immunoelectrophoresis in the identification of *Streptococcus pneumoniae* in clinical isolates. J. Clin. Microbiol. **3**:173–177.
16. **Spencer, R. C., and M. A. Savage.** 1976. Use of counter and rocket immunoelectrophoresis in acute respiratory infections due to *Streptococcus pneumoniae*. J. Clin. Pathol. **29**:187–190.
17. **Trollfors, B., E. Berntsson, B. Eljefors, and B. Kaijser.** 1979. CIE of sputum and blood for the diagnosis of chest infections caused by pneumococci or *Haemophilus influenzae*. Scand. J. Infect. Dis. **11**:31–39.
18. **Tugwell, P., and B. M. Greenwood.** 1975. Pneumococcal antigen in lobar pneumonia. J. Clin. Pathol. **28**:118–123.
19. **Wicher, K., C. Kalinka, P. Mlodozeniec, and N. Rose.** 1982. Fluorescent antibody technic used for identification and typing of *Streptococcus pneumoniae*. Am. J. Clin. Pathol. **77**:72–77.
20. **Yurchah, A., and R. Austrian.** 1966. Serologic and genetic relationship between pneumococci and other respiratory streptococci. Trans. Assoc. Am. Phys. **79**:368–375.