JOURNAL OF CLINICAL MICROBIOLOGY, July 1985, p. 111–115 Vol. 22, No. 1
0095-1137/85/070111-05$02.00/0
Copyright © 1985, American Society for Microbiology

# Detection of C Polysaccharide in *Streptococcus pneumoniae* in the Sputa of Pneumonia Patients by an Enzyme-Linked Immunosorbent Assay

HANS HOLMBERG,[1]* TORD HOLME,[2] AUD KROOK,[3] THOMAS OLSSON,[4] LENNART SJÖBERG,[1] AND ANN-MARGRET SJÖGREN[2]

*Departments of Clinical Microbiology and Immunology[1] and Infectious Diseases,[3] Örebro Medical Center Hospital, S-701 85 Örebro, Department of Bacteriology, Karolinska Institute, S-104 01 Stockholm,[2] and LKB Produkter AB, S-161 26 Bromma,[4] Sweden*

Received 26 November 1984/Accepted 27 March 1985

**The pneumococcal C polysaccharide (PnC) is species specific and believed to be a cell wall component of all pneumococcal types. A sandwich enzyme-linked immunosorbent assay (ELISA) for detection of PnC in sputa has been developed by using a monoclonal antiphosphorylcholine antibody and a polyclonal rabbit anti-PnC antiserum in the test system. A 1-year study of adult hospitalized patients with community-acquired pneumonia was performed. A total of 147 patients with clinical and radiological evidence for pneumonia were accepted for the study. Of these, 105 patients provided a sputum sample upon admission to the ward. The sputa were cultured semiquantitatively as well as tested for the presence of antigen. Of the sputum samples from patients with *Streptococcus pneumoniae*, 27 of 33 (accounting for a sensitivity of 82%) were positive in the ELISA test. Of the sputum samples from patients with pneumonia of some other known or suspected etiology, 32 of 34 (accounting for a specificity of 94%) were negative. In addition, 7 sputum samples from 31 patients with pneumonia of unknown etiology were positive. The ELISA test described here is in our opinion a sensitive and specific test for detecting PnC from *S. pneumoniae* in sputa from patients with untreated pneumonia.**

*Streptococcus pneumoniae* accounts for most cases of bacterial pneumonia, with an incidence of 1 to 5 cases per 1,000 persons per year in the United States (1). Blood culture can be used to establish a diagnosis of pneumococcal pneumonia. However, blood cultures are positive in only about one-fourth of the cases (2). Other diagnostic methods used are semiquantitative cultures of homogenized sputa (3) and nasopharyngeal swab cultures (12), but these methods sometimes fail, especially in patients treated with antibiotics. Bacterial culture requires at least 24 h for diagnosis. Since there is a need for more rapid diagnosis, methods for detection of soluble antigens in sputa have been developed. The methods used are immunoelectroosmophoresis (7, 16, 21, 27, 29) and coagglutination (8, 14). The diagnostic sera used in these investigations have been a polyvalent antiserum, Omniserum, containing anticapsular antibodies against the 83 known types of pneumococci and, in some studies, 9 pooled sera and 46 typed sera. All of these sera are produced by the State Serum Institute, Copenhagen, Denmark (18).

With the sensitive methods used for antigen detection, antigens from alpha-hemolytic streptococci have been shown to react with Omniserum as well as with type sera (26, 31). These findings indicate a risk for false-positive reactions in tests of sputa or other specimens which may be contaminated with alpha-hemolytic streptococci.

The pneumococcal C polysaccharide (PnC) is species specific and believed to be a cell wall component of all pneumococcal types. The complete structure of PnC has been reported by Jennings et al. (11), who used methylation analysis and nuclear magnetic resonance spectroscopic techniques. PnC is composed of a pentasaccharide repeating unit with a ribitol phosphate linkage. These workers found identical PnC in the cell wall of strains representing four capsular types as well as from a rough strain derived from a type 2 organism. PnC contains phosphorylcholine (5, 11, 23), and antibodies reactive with the phosphorylcholine determinant are induced in mice and rabbits after immunization with pneumococci, especially when noncapsular mutants are used (6, 25). Antibodies reactive with phosphorylcholine can also be induced by other bacteria, including group O and H streptococci, *Lactobacillus acidophilus*, and *Proteus morganii* (22). Murine monoclonal antiphosphorylcholine antibodies have been produced in several studies (6, 9, 25, 28). In our efforts to improve the laboratory diagnosis of pneumococcal pneumonia, we developed a method for the detection of PnC in sputa from patients with pneumonia. The technique used was a modified sandwich enzyme-linked immunosorbent assay (ELISA) with a monoclonal antiphosphorylcholine antibody and a polyclonal rabbit anti-PnC antiserum in the test system.

## MATERIALS AND METHODS

**Patients and samples.** A 1-year study of adult hospitalized patients with community-acquired pneumonia was performed at the Department of Infectious Diseases, Örebro Medical Center Hospital, Örebro, Sweden. A total of 147 patients with clinical and radiological evidence for pneumonia were accepted for the study. Upon admission to the ward (day 0), 105 patients provided a sputum sample. On the following morning (day 1), 94 of these patients provided a second sputum sample. Twenty-four patients provided a sputum sample on day 1 only. Altogether, 129 patients provided 223 sputum samples from day 0 or day 1 (or both days), which were cultured as well as tested for the presence of antigen. Twenty-two patients (15%) had been treated with

* Corresponding author.

antibiotics before admission with little or no clinical effect. Upon admission to the hospital, antibiotic treatment was started after specimens of blood, sputum, nasopharynx, and urine had been taken.

**Bacteriological procedures.** Three samples for aerobic (BACTEC 8B) and anaerobic (BACTEC 7D) blood culture (Johnston Laboratories, Inc., Towson, Md.) were drawn from each patient and incubated for at least 1 week. Nasopharynx swab culture was taken at day 0 and day 1. The specimens were cultured on hematin agar (horse blood, 8.5%) and bovine blood agar (10%) with gentian violet (3 μg/ml); both plates were incubated in moist air with 5% $CO_2$.

Sputum culture was performed in a semiquantitative manner. The samples were homogenized with an equal volume of Sputolysin (Calbiochem Behring, La Jolla, Calif.), shaken on a Vortex mixer for 5 min, and then incubated at room temperature for another 15 min. Of these partially or totally homogenized sputa, 10-μl portions were taken with a loop, cultured on hematin agar with a 50-μg oleandomycin disk, and incubated in moist air with 5% $CO_2$. In this procedure one colony represents $2 \times 10^2$ CFU/ml. Another sample was diluted 1:100 with nutrient broth (Oxoid Ltd., Basingstoke, United Kingdom). Portions (1 μl) of this dilution were cultured on hematin agar as described above, on bovine blood agar with gentian violet provided with two paper disks containing 0.2 IU of bacitracin and 6 μg of optochin, respectively, incubated in moist air with 5% $CO_2$, and anaerobically (GasPak; BBL Microbiology Systems, Cockeysville, Md.) on brain heart infusion-blood agar (5% bovine blood) supplemented with yeast extract, vitamin K, and hemin (4 μg/ml) provided with an optochin paper disk. With this dilution one colony represents $2 \times 10^5$ CFU/ml. All sputum samples were Gram stained after homogenization and examined microscopically. The leukocyte/squamous epithelial cell ratio and the dominating bacteria were estimated according to the method of Kalin et al. (15).

Isolates of suspected *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, and enteric gram-negative rods were subcultured if they exceeded $2 \times 10^5$ CFU/ml in the sputum culture. Suspect *Branhamella catarrhalis* organisms were noted only if they dominated the culture. Pneumococci were identified by their optochin susceptibility. Strain typing was kindly performed by J. Henrichsen, Statens Serum Institut, Copenhagen, Denmark. *Staphylococcus aureus* was identified by DNase positivity and, if still unclear, by the coagulase test. *H. influenzae* was identified with V, X, and VX factor disk tests (Biodisk, Solna, Sweden). Enteric gram-negative rods were identified by a microtube system (API System SA, Montalieu, Vercieu, France). *B. catarrhalis* was identified by colony morphology and oxidase positivity.

**Procedure for antigen detection. (i) Preparation of PnC.** The PnC was prepared from a pneumococcal C mutant strain, CSR-SCS-2 clone 1 (kindly provided by J. Henrichsen) (20, 25). This strain is reported to carry a small capsule consisting of C polysaccharide (4, 24). The pneumococci were grown with slow magnetic stirring for 11 h at 37°C in 1-liter portions of brain heart infusion medium (Difco Laboratories, Detroit, Mich.). Each culture was tested for bacterial contamination. The supernatants were removed by centrifugation at 4°C for 30 min at $15{,}000 \times g$. The bacteria were suspended in distilled water at a concentration of 20 g (dry weight) per liter and extracted with an equal volume of 90% phenol at 68°C for 15 min (30). After dialysis for 24 h against running tap water, the remaining material was freeze-dried. The protein content of the polysaccharide preparation was 0.17 mg/mg as measured by the method of Lowry et al. (17).

**(ii) Polyclonal and monoclonal antibodies against PnC.** Polyclonal antiserum was raised in rabbits by immunization with *S. pneumoniae* (CSR-SCS-2) and partially purified by precipitation with 50% ammonium sulfate. The resulting precipitate was suspended to the original volume in phosphate-buffered saline (PBS) containing 0.02% $NaN_3$, 0.5% bovine serum albumin (BSA), and 0.05% Tween 20 (PBS-BSA-Tween). It was used in a dilution of 1/1,000 in the same buffer and was stable in concentrated form at −20°C for several months.

Murine monoclonal antibodies were produced by using the same strain (CSR-SCS-2) of *S. pneumoniae* and the hybridoma technique described by Nowinsky et al. (19). Hybridomas were screened for PnC antibody production by using microtiter plates (M 129A; Dynatech Laboratories, Inc., Alexandria, Va.) coated with PnC. A clone producing immunoglobulin M (IgM) antibodies was selected for use in the present immunoassay. The specificity of these antibodies was ascertained by the inhibition caused by PnC or phosphorylcholine.

Monoclonal antibodies (IgM) were precipitated from ascites fluid by dialysis against deionized water at 4°C overnight (10). The precipitate was dissolved in PBS containing 1 M NaCl at 22°C and further purified by high-pressure preparative gel chromatography on an LKB Ultropac TSK-G4000 SW column (7.5 by 300 mm) by using the LKB 2150 high-pressure liquid chromatography pump with a coupled UV detector and potentiometric recorder (LKB Produkter AB, Bromma, Sweden).

**(iii) Patient sample examination.** Sputum specimens treated as described above were stored frozen at −20°C until assayed for PnC. The samples (0.2 ml) were placed on a boiling water bath for 3 min followed by 1/50 dilution in PBS-BSA-Tween (final dilution, 1/100) before the assay. The boiling and dilution was necessary to remove material in sputum which interfered with the assay.

The assay of PnC was performed in 96-well microtiter plates (Dynatech M 129A; Dynatech Laboratories) coated with 0.1 ml of monoclonal antibodies (5 μg/ml) at pH 6.0 in 50 mM $PO_4^{3-}$ at 22°C overnight. To avoid possible variations in results due to temperature effects, the marginal rows were not used. Volumes of 0.1 ml per well and a temperature of 37°C for 1 h were used in all incubations for this assay. The antibody activity in these plates was stable for several weeks after storage at 4°C. Before use the plates were washed three times with PBS-Tween buffer and incubated for 1 h with PBS-BSA-Tween buffer to minimize unspecific adsorption in subsequent steps. The same washing procedure was repeated after each incubation. In the next step, diluted samples were incubated in duplicate and then incubated with polyclonal rabbit PnC antibodies. The amount of adsorbed rabbit antibodies was finally quantitated with swine anti-rabbit IgG conjugated to alkaline phosphatase diluted 1/100 in PBS-Tween-BSA (Orion Diagnostics, Helsinki, Finland). The enzyme activity was determined with *p*-nitrophenylphosphate (1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) as the substrate in 10% diethanolamine hydrochloride (pH 9.8), and the formation of *p*-nitrophenol was measured at 405 nm with a Titertek Multiscan MC spectrophotometer (Flow Laboratories, Inc., McLean, Va.). The enzyme activity was expressed as the optical density at 405 nm per hour. An optical density of 0.100, i.e., at least twice the background value, was chosen as the cutoff value for a positive or negative test.

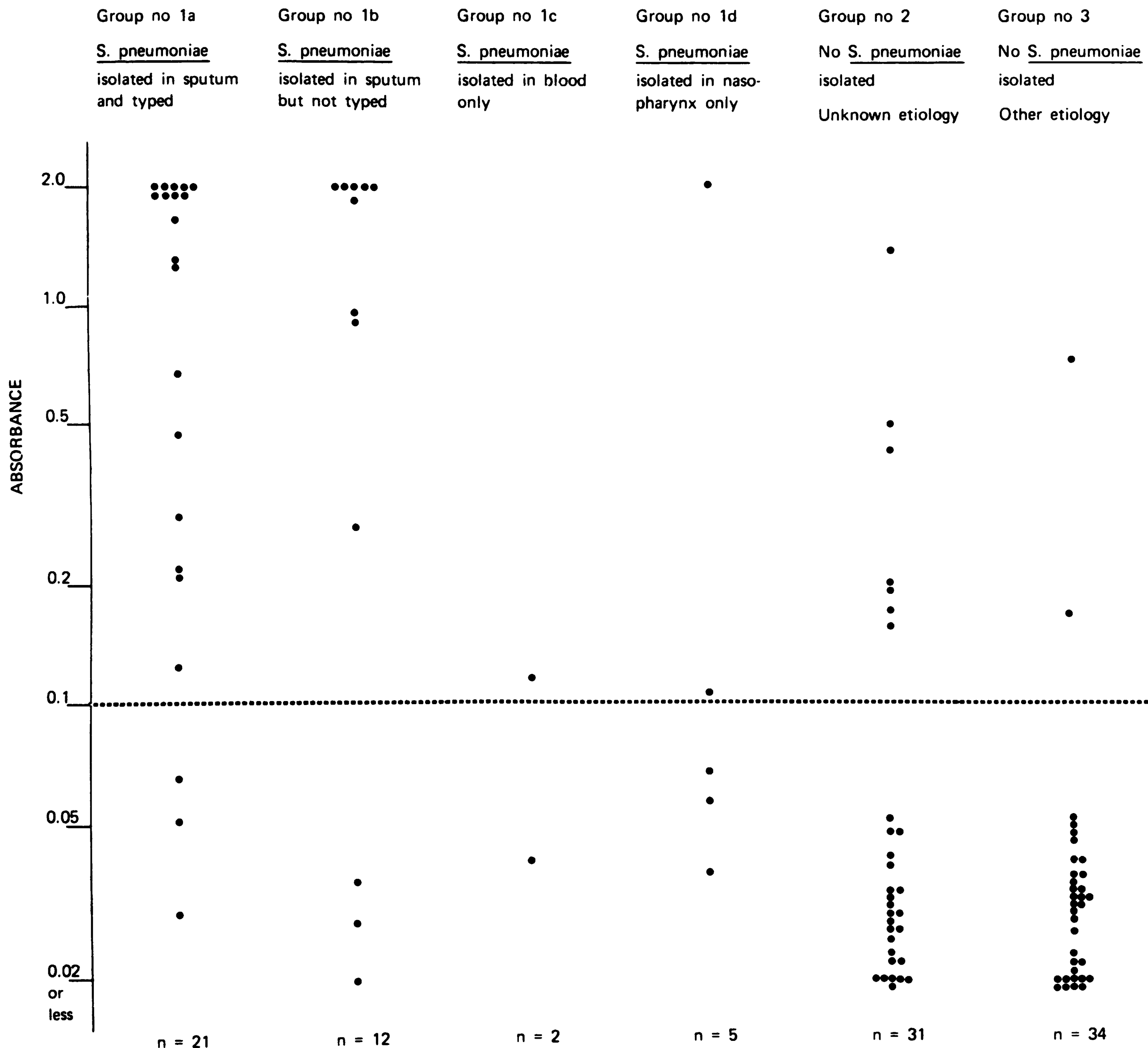


FIG. 1. Absorbance values in the ELISA test for detection of PnC in sputum. Results are from 105 sputum samples obtained from 105 patients with radiologically verified pneumonia. Absorbance values below 0.1 are considered negative.

## RESULTS

The absorbance values in the ELISA test of the 105 sputum samples delivered upon admission to the ward are shown in Fig. 1. Group 1a includes 21 sputum samples from which *S. pneumoniae* was isolated and later typed (Table 1). From four of the sputum samples in this group, one additional bacterial species was isolated in quantitites indicating a possible significance as follows: two *H. influenzae*, one *Staphylococcus aureus*, and one *Escherichia coli*. Group 1b represents another 12 sputum samples from which *S. pneumoniae* was isolated but not stored and typed. From one sputum sample in this group *E. coli* was isolated, and in one sputum sample *H. influenzae* was found in addition to *S. pneumoniae*. Group 1c represents sputum samples from two patients with *S. pneumoniae* isolated in blood only, and group 1d represents sputum samples from five patients with *S. pneumoniae* isolated in the nasopharynx only. Group 2 represents sputum samples from 31 patients with pneumonia of unknown etiology. Group 3 represents sputum samples from 34 patients with pneumonia of some other known or suspected etiology.

The results of antigen detection in the 105 sputum samples are summarized in Table 1. Of 33 samples, 27 (81.8%) with *S. pneumoniae* isolated were positive in the ELISA test. In 22 of these 33 patients, pneumococci could not be isolated in sputa the following day (day 1). Of these 22 sputum samples, 16 were antigen positive on day 0 and 7 were still positive on day 1.

The results of culture and antigen detection in purulent and nonpurulent sputa are recorded in Table 2. The 105 sputum samples collected upon admission to the ward are represented. A positive antigen detection test was obtained in 87% (20 of 23) of the purulent sputum samples in which *S. pneumoniae* had been isolated, compared with 70% (7 of 10) of the nonpurulent sputum samples.

TABLE 1. Pneumococcal antigen detection by ELISA in 105 sputum samples from 105 patients provided upon admission to the ward

| Group | Description | No. of sputum samples[a] + | No. of sputum samples[a] − | Positive test (%) |
|---|---|---|---|---|
| 1a | *S. pneumoniae* isolated in sputum; typed strains[b] | 18 (0) | 3 (0) | 85.7 |
| 1b | *S. pneumoniae* isolated in sputum but not typed | 9 (0) | 3 (0) | 75.0 |
| 1c | *S. pneumoniae* isolated in blood and not in sputum | 1 (0)[c] | 1 (0)[d] | |
| 1d | *S. pneumoniae* isolated in nasopharynx only | 2 (0) | 3 (0) | |
| 2 | No *S. pneumoniae* isolated in sputum, blood, or nasopharynx; patients with pneumonia of unknown etiology | 7 (2) | 24 (7)[e] | 22.6 |
| 3 | No *S. pneumoniae* isolated in sputum, blood, or nasopharynx; patients with pneumonia of other known or suspected etiology | 2 (0) | 32 (5) | 5.9 |

[a] Numbers of patients given antibiotic treatment before admission to the ward are given in parentheses.
[b] Types (number of isolates when greater than one is given in parentheses): 3 (10), 6A (2), 7F (2), 14, 19A, 19F (2), 20, and 23F (2).
[c] Type 8 isolated in blood.
[d] Type 19A isolated in blood.
[e] In one patient *S. pneumoniae* was isolated and the ELISA test was positive on day 1.

## DISCUSSION

The sensitivity (85.7%) of the pneumococcal antigen test in the day-0 samples with typed pneumococci is promising (Table 1). Types 3, 14, and 19A were isolated from the three sputum samples with a negative antigen test. However, in tests with suspensions of these three strains in the ELISA system described here, they were all positive. The amount of PnC in the cell walls of the bacteria in the sputum samples or the number of bacteria may have been too low to be detected by the test. Of 40 patients with *S. pneumoniae* isolated in their blood, sputum, or nasopharyngeal culture, 30 (75.0%) were positive in the antigen test (Table 1).

We separated the groups with typed and untyped pneumococci. In our experience there are some difficulties in the diagnosis of pneumococci with an optochin paper disk due to borderline sensitivity for optochin in a few strains of alpha-hemolytic streptococci. The sensitivity of the ELISA test in the untyped group, 75.0% compared with 85.7% in the typed group, might indicate that some of these untyped "pneumococci" were alpha-hemolytic streptococci. This demonstrates the importance of a reliable procedure for bacteriological diagnosis in studies of immunological methods.

The specificity of the test was good and as high as 94.1% when the group of 34 patients with pneumonia of other known or suspected etiology was used as the control group (Table 1). The two positive sputum samples in this group had absorbance values of 0.163 and 0.720 (Fig. 1), the latter well above the cutoff value of 0.100. In this last patient, who had chronic bronchitis due to smoking, *H. influenzae* had been isolated in the nasopharynx on day 0 as well as day 1, but the cultures from blood and sputa and the serological tests were negative. It is possible that the positive ELISA test result is true, indicating a double infection with *S. pneumoniae* and *H. influenzae*. However, an immunological cross-reaction between strains of *H. influenzae* and the PnC cannot be ruled out. The other patient had negative cultures from blood, sputa, and the nasopharynx, but showed a fourfold increase of IgM and IgG antibodies to *Legionella pneumophila* type 1 in the convalescent serum. Antigenic similarities or a double infection might also be the reason for the positive antigen test in this patient.

The seven positive sputum samples, representing seven patients with pneumonia of unknown etiology (Table 1), originated from patients with no potentially pathogenic bacteria isolated in blood, sputa, or nasopharynx and with negative serology. The absorbance values of these seven sputum samples, which were between 0.160 and 1.360 with four samples not exceeding 0.200, differ in this respect from the often very high absorbance values from sputum samples with *S. pneumoniae* isolated, thus indicating a lower antigen concentration in these samples. These seven patients were all cured by treatment with penicillin, and from a clinical point of view there were no objections against a possible pneumococcal etiology.

Antibiotic treatment was started at the day of admission in all of our patients. As a result, sputa obtained the following day were often culture negative. Only 7 (31.8%) of the 22 patients with *S. pneumoniae* isolated in their sputa on day 0 but not day 1 were antigen positive on day 1, indicating a rapid decrease in the amount of antigen after the initiation of antibiotic treatment. This frequency is low compared with the results of Kalin and Lindberg using Omniserum and immunoelectroosmophoresis (13). They obtained a positive sputum test in 47.1% of the posttreatment samples from patients with pneumococcal pneumonia, which may indicate that the capsular antigens remain in sputum longer than does the PnC. This finding may also be a direct effect of penicillin since it is an antibiotic interfering with cell wall synthesis and PnC is a cell wall component. Of the 105 patients providing a sputum sample upon admission, 14 had been treated with antibiotics before their arrival to the ward. None of the 33 patients with *S. pneumoniae* isolated in their sputa, compared with 9 of the 31 (29.0%) patients with pneumonia of unknown etiology, had received antibiotics

TABLE 2. Comparison between bacterial culture and pneumococcal antigen ELISA results in 105 sputum samples from 105 patients provided upon admission to the ward

| Result by bacterial culture | Results by antigen ELISA in: Purulent sputum samples ($n$ = 63) Positive | Purulent sputum samples ($n$ = 63) Negative | Nonpurulent sputum samples ($n$ = 42) Positive | Nonpurulent sputum samples ($n$ = 42) Negative |
|---|---|---|---|---|
| Positive | 20 | 3[a] | 7 | 3[b] |
| Negative | 8[c] | 32 | 4[d] | 28 |

[a] Type 19A and two untyped strains.
[b] Types 3 and 14 and one untyped strain.
[c] Samples representing the following patients: one with positive blood culture for *S. pneumoniae*, five with pneumonia of unknown etiology, one with *H. influenzae* in nasopharynx culture, and one with positive serology for *L. pneumophila*.
[d] Samples representing the following patients: two with *S. pneumoniae* isolated in nasopharynx culture and two with pneumonia of unknown etiology.

(Table 1). In the latter group only two of the seven patients with a positive antigen test on day 0 had been treated with antibiotics. This is in agreement with the observation of a rapid disappearance of antigen after antibiotic treatment, indicating that the ELISA test is less sensitive in patients treated with antibiotics.

The frequency of a positive antigen test in relation to positive culture in the nonpurulent sputa from day 0 (70.0%, 7 of 10) is lower than that obtained with the purulent sputa (87.0%, 20 of 23). This is not surprising since the sputa from the latter group contain large amounts of leukocytes with many bacteria and so should contain a high amount of antigen. The results indicate, however, that all sputa regardless of the degree of purulency should be tested for the presence of antigen.

The ELISA test described here is in our opinion a sensitive and specific test for detecting PnC from *S. pneumoniae* in sputa from untreated patients with pneumonia. For use in routine laboratory diagnosis of pulmonary infections it would be desirable to simplify the test procedure to shorten the time required from the present 4 to 5 h to 1 to 2 h.

## ACKNOWLEDGMENTS

This work was supported by the Swedish Board for Technical Development.

We thank Elisabeth Mansfeldt for skillful laboratory assistance.

## LITERATURE CITED

1. **Austrian, R.** 1974. Pneumococcal infection. Prev. Med. 3:443–445.
2. **Austrian, R., and J. Gold.** 1964. Pneumococcal bacteremia with especial reference to bacteremic pneumococcal pneumonia. Ann. Intern. Med. 60:759–776.
3. **Bartlett, J. G., and S. M. Finegold.** 1978. Bacteriology of expectorated sputum with quantitative culture and wash technique compared to transtracheal aspiration. Am. Rev. Respir. Dis. 117:1019–1027.
4. **Bornstein, D. L., G. Schiffman, H. P. Bernheimer, and R. Austrian.** 1968. Capsulation of pneumococcus with soluble C-like ($C_s$) polysaccharide. J. Exp. Med. 128:1385–1400.
5. **Brundish, D. E., and J. Baddiley.** 1968. Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. Biochem. J. 110:573–581.
6. **Claflin, J. L., S. Hudak, and A. Maddalena.** 1981. Anti-phosphocholine hybridoma antibodies: direct evidence for three distinct families of antibodies in the murine response. J. Exp. Med. 153:352–364.
7. **Coonrod, J. D., and M. W. Rytel.** 1973. Detection of type-specific pneumococcal antigens by counter immunoelectrophoresis. J. Lab. Clin. Med. 81:778–786.
8. **Edwards, E. A., and J. D. Coonrod.** 1980. Coagglutination and counterimmunoelectrophoresis for detection of pneumococcal antigens in the sputum of pneumonia patients. J. Clin. Microbiol. 11:488–491.
9. **Gearhart, P. J., N. D. Johnson, R. Douglas, and L. Hood.** 1981. IgG antibodies to phosphorylcholine exhibit more diversity than their IgM counterparts. Nature (London) 291:29–34.
10. **Heide, K., and H. G. Schwick.** 1978. Salt fractionation of immunoglobulins, p. 7.1–7.11. *In* D. M. Weir (ed.), Handbook of experimental immunology, vol. 1. Blackwell Scientific Publications, London.
11. **Jennings, H. J., C. Lugowski, and N. M. Young.** 1980. Structure of the complex polysaccharide C substance from *Streptococcus pneumoniae* type 1. Biochemistry 19:4712–4719.
12. **Kalin, M.** 1982. Bacteremic pneumococcal pneumonia: value of culture of nasopharyngeal specimen and examination of washed sputum specimens. Eur. J. Clin. Microbiol. 1:394–396.
13. **Kalin, M., and A. A. Lindberg.** 1983. Diagnosis of pneumococcal pneumonia: a comparison between microscopic examination of expectorate, antigen detection and cultural procedures. Scand. J. Infect. Dis. 15:247–255.
14. **Kalin, M., A. A. Lindberg, and E.-H. Olausson.** 1982. Diagnosis of pneumococcal pneumonia by coagglutination and counter immunoelectrophoresis of sputum samples. Eur. J. Clin. Microbiol. 2:91–96.
15. **Kalin, M., A. A. Lindberg, and G. Tunevall.** 1983. Etiological diagnosis of bacterial pneumonia by Gram stain and quantitative culture of expectorates. Scand. J. Infect. Dis. 15:153–160.
16. **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.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
18. **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.
19. **Nowinski, R. C., M. E. Lostrom, M. R. Tam, M. R. Stone, and W. N. Burnette.** 1979. The isolation of hybrid cell lines producing monoclonal antibodies against the p15 (E) protein of ecotropic murine leukemia viruses. Virology 93:111–126.
20. **Pedersen, K. P., J. Henrichsen, U. S. Sörensen, and J. L. Nielsen.** 1982. Anti-C-carbohydrate antibodies after pneumococcal vaccination. Acta Pathol. Microbiol. Scand. Sect. C 90:353–355.
21. **Perlino, C. A., and J. A. Schulman.** 1976. Detection of pneumococcal pneumonia by counter immunoelectrophoresis. J. Lab. Clin. Med. 87:496–502.
22. **Potter, M.** 1977. Antigen-binding myeloma proteins of mice. Adv. Immunol. 25:141–211.
23. **Poxton, I. R., E. Tarelli, and J. Baddiley.** 1978. The structure of C-polysaccharide from the walls of *Streptococcus pneumoniae*. Biochem. J. 175:1033–1042.
24. **Schiffman, G., D. L. Bornstein, and R. Austrian.** 1971. Capsulation of pneumococcus with soluble cell wall-like polysaccharide. J. Exp. Med. 134:600–617.
25. **Skov Sørensen, U. B., R. Agger, J. Bennedsen, and J. Henrichsen.** 1984. Phosphorylcholine determinants in six pneumococcal capsular polysaccharides detected by monoclonal antibody. Infect. Immun. 43:876–878.
26. **Sottile, M. I., and M. W. Rytel.** 1975. Application of counterimmunoelectrophoresis in the identification of *Streptococcus pneumoniae* in clinical isolates. J. Clin. Microbiol. 2:173–177.
27. **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.
28. **Szu, S. C., S. Clarke, and J. B. Robbins.** 1983. Protection against pneumococcal infection in mice conferred by phosphocholine-binding antibodies: specificity of the phosphocholine binding and relation to several types. Infect. Immun. 39:993–999.
29. **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.
30. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. 5:83–91.
31. **Yurchah, A., and R. Austrian.** 1966. Serologic and genetic relationship between pneumococci and other respiratory streptococci. Trans. Assoc. Am. Phys. 79:368–375.