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Quantitation of Pneumococcal C Polysaccharide in Sputum Samples from Patients with Presumptive Pneumococcal Pneumonia by Enzyme Immunoassay

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Although the Gram stain and culture of expectorated sputum are considered standard methods for the diagnosis of presumptive pneumococcal pneumonia, these methods remain relatively insensitive and nonspecific. We developed an enzyme immunoassay (EIA) for the quantitation of pneumococcal C polysaccharide (PnC) in the sputum of patients with presumptive pneumococcal pneumonia. Of 34 patient sputum samples collected within 24 h of the first radiographic report of pneumonia, 12 grew *Streptococcus pneumoniae* on culture. By using a cutoff point of 0.5 μg of PnC per ml of sputum, all 12 specimens were positive (sensitivity, 100%) by EIA. PnC levels ranged from 1.43 to 57.53 μg/ml. Blood samples from 18 of the 34 patients were cultured. *S. pneumoniae* grew in the culture of a blood sample from one patient, whose sputum also had the highest PnC level. Of 22 sputum samples from patients with pneumonia that did not grow *S. pneumoniae*, two were positive by EIA (specificity, 90.1%). Sputa from both patients had low levels of PnC (2.7 and 4.5 μg/ml), and both patients had received antibiotics before sputum collection. The positive predictive value of the quantitative EIA was 85.7%. Quantitation of PnC has the potential for improving the accuracy of sputum examination for *S. pneumoniae*, monitoring disease severity and the effectiveness of antibiotic therapy, and differentiating between those patients with invasive pneumococcal disease and those who are carriers of *S. pneumoniae*.

Culture and Gram stain of expectorated sputum remain the time-honored methods for microbiologic evaluation of pneumococcal pneumonia, despite numerous studies indicating discrepancies between culture results and the identification of the etiologic agent in cultures of blood (1, 12, 14). Fewer than 50% of expectorated sputum samples processed by the methods used by clinical laboratories yield results that contribute to a diagnosis because of problems associated with upper airway colonization with potentially pathogenic organisms (1). Alternative methods have been used to increase the diagnostic accuracy of sputum examination in patients with suspected pneumococcal pneumonia. These have included culturing of sputum after washing the sputum specimen and quantitation of pathogenic bacteria after dilution of the specimen (2, 6). However, such methods have not proven practical for the routine screening of large numbers of sputum samples. Methods potentially more practical for the rapid processing of large numbers of specimens have included the use of coagglutination, latex particle agglutination, and counterimmunoelectrophoresis to detect pneumococcal capsular polysaccharide antigens in sputum specimens (4, 10, 13, 17, 19, 23). However, these assays have not been shown to be uniformly sensitive or specific. This may be because the polyvalent antiserum used contains antibody to all 83 pneumococcal polysaccharide serotype antigens. Since each polysaccharide antigen may vary in immunogenicity, the antiserum produced by immunization and used in these detection systems may fail to detect the less immunogenic pneumococcal serotypes. In addition, cross-reactions between polyvalent antisera and certain strains of viridans group streptococci and *Haemophilus influenzae* may occur. These cross-reactions may lead to false-positive reactions in sputum contaminated with these bacteria (4, 7, 18).

Recently, we reported the results of an evaluation of a commercial prototype enzyme immunoassay (EIA) which detected pneumococcal C polysaccharide (PnC) in the sputum of patients with presumptive pneumococcal pneumonia (15). PnC is a phosphocholine-containing polysaccharide in the pneumococcal cell wall, is derived from teichoic acid, and is common to all pneumococcal serotypes (3, 9, 21). This assay system demonstrated a diagnostic sensitivity of 100% and a specificity of 78.2%. To our knowledge, this assay was never marketed. In this report, we describe the development and evaluation of a quantitative EIA for the detection of PnC in sputum of patients with presumptive pneumococcal pneumonia.

MATERIALS AND METHODS

Patients and samples. The patient population and sample collection methods have been described as part of a previous study (15). Briefly, sputum samples were collected from patients with a suspected clinical diagnosis of pneumonia who were seen at the Alaska Native Medical Center during a 5-month period from February through June 1987. All patients underwent a chest radiograph. Sputum samples were collected within 24 h of the radiograph. Sputum samples were plated onto Trypticase soy agar (BBL, Becton Dickinson, Cockeysville, Md.) with 5% sheep blood and

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onto eosin-methylene blue agar. Blood agar plates were incubated in the presence of 5% CO_2 at 35°C for 24 h. Pneumococcal isolates were identified by their susceptibilities to a 5 μg optochin disc. *H. influenzae* was grown on blood agar adjacent to a streak of *Staphylococcus aureus* and was identified by Gram staining and other biochemical characteristics. Other respiratory pathogens were identified by cultural and biochemical characteristics. The normal flora was defined as those organisms that are normally present in the upper respiratory tract. Sputum samples were stored frozen at −30°C.

EIA. Sputum samples were thawed rapidly at 37°C and were homogenized by adding an equal volume of sputolysin (Calbiochem-Behring, La Jolla, Calif.), vortexing them for 5 min, and incubating them at room temperature for 15 min. Homogenized sputum samples were diluted 1:25 in phosphate-buffered saline (PBS), boiled for 4 min, and allowed to cool. Microplates (Costar strips) were coated overnight at room temperature with 100 μl of a 1/3,200 dilution of mouse immunoglobulin G3 monoclonal antibody to phosphocholine (22) in phosphate buffer (pH 6.0). Plates were washed six times with PBS with 0.05% Tween 20 and were blocked for 60 minutes at room temperature with 0.5% bovine serum albumin in PBS. After washing, 100 μl of diluted sputum was added in duplicate to microplate wells coated with anti-PnC (monoclonal antibody). Purified PnC (provided by Gerald Schiffman, State University of New York) was diluted twofold in PBS to provide a standard curve, which ranged from 0.025 to 25 ng of PnC per ml, and 100 μl of each standard was added to duplicated wells containing anti-PnC (monoclonal antibody). Plates containing both sputum samples and standards were sealed and were incubated for 2 h at 37°C. After incubation, plates were washed six times in PBS–0.05% Tween 20. Anti-PnC (rabbit polyclonal; Dako Corporation, Carpinteria, Calif.) was diluted to 1/1,000 in 0.5% bovine serum albumin in PBS, and 100 μl was added to all wells. Plates were incubated at 37°C for 60 min, after which plates were again washed, and 100 μl of alkaline phosphatase-labeled anti-rabbit immunoglobulin G (goat; Sigma, St. Louis, Mo.) diluted 1/1,000 in 0.5% bovine serum albumin–PBS was added to all wells. After this, plates were incubated for 60 min at 37°C and washed, and 100 μl of substrate (*para*-nitrophenol phosphate [1 mg/ml] in 50 mM diethanolamine [pH 9.8]) was added to all wells. Plates were then incubated for 30 min and read at A_{410} by using a Dynatech MR700 plate reader. Standard curves were established by using an iterative robust fitting procedure, as described by Tiede and Pagano (20), to identify aberrant (outlying) observations by using a BASIC program as detailed by Plikaytis et al. (16). The final standard curve was estimated by ordinary unweighted least-squares analysis. PnC levels in sputum were then interpolated from this curve from their respective optical density reading.

Levels of PnC in standard suspensions of both pneumococcal and nonpneumococcal isolates were measured by EIA. Bacterial isolates were grown overnight at 35°C on Trypticase soy agar supplemented with 5% sheep blood. Suspensions containing 10^8 cells per ml (McFarland no. 0.5) were prepared in PBS. Suspensions were diluted from 1/10 to 1/10,000 in PBS and were boiled for 4 min before we tested them for PnC as described above.

RESULTS

PnC levels in standard suspensions of pneumococcal and nonpneumococcal bacteria. PnC levels in standardized suspensions (10^8 organisms per ml) of 21 pneumococcal serotypes, 38 other bacterial isolates, and 2 yeast isolates were measured by the EIA (Table 1). All pneumococcal isolates tested showed high levels of PnC (0.81 to 15.0 μg/ml). The amounts of PnC produced varied by serotype. Four nonpneumococcal isolates had detectable PnC levels, ranging from 0.004 to 0.020 μg/ml. All other nonpneumococcal isolates tested had PnC levels of ≤0.002 μg/ml.

TABLE 1. Detection of PnC in pneumococcal and nonpneumococcal bacterial cultures by EIA[a]

Culture (serotype)	Range (μg/ml)
Streptococcus pneumoniae (3, 4, 6, 7F, 9V, 10A, 12F, 14, 15, 17F, 18C, 19A, 19F, 20, 33)	0.810–3.65
Streptococcus pneumoniae (8, 11A, 22F)	5.20–5.50
Streptococcus pneumoniae (1, 9N, 23)	10.25–15.0
Staphylococcus haemolyticus, *Streptococcus pyogenes*, *Staphylococcus aureus* (Cowan I), *Candida albicans*, *Proteus vulgaris*	0.004–0.020
Streptococcus group A type I	0–0.002
Streptococcus group A type II	0–0.002
Streptococcus group B type 1a	0–0.002
Streptococcus group B type II	0–0.002
Streptococcus group B type II	0–0.002
Streptococcus group C equis	0–0.002
Streptococcus group D duran	0–0.002
Streptococcus group F	0–0.002
Streptococcus group G	0–0.002
Streptococcus faecalis	0–0.002
Streptococcus bovis ATCC 9809	0–0.002
Streptococcus mitis viridans	0–0.002
Streptococcus intermedius A viridans	0–0.002
Streptococcus intermedius-mitis I viridans	0–0.002
Streptococcus sanguis viridans	0–0.002
Staphylococcus aureus ATCC 25923	0–0.002
Staphylococcus saprophyticus	0–0.002
Staphylococcus epidermidis ATCC 12228	0–0.002
Staphylococcus simulans	0–0.002
Staphylococcus cohnni	0–0.002
Staphylococcus warneri	0–0.002
Staphylococcus xylosus ATCC 29971	0–0.002
Haemophilus influenzae (b)	0–0.002
Escherichia coli ATCC 25922	0–0.002
Enterobacter cloacae ATCC 23355	0–0.002
Klebsiella pneumoniae ATCC 13883	0–0.002
Serratia marcescens ATCC 8100	0–0.002
Salmonella typhimurium ATCC 14028	0–0.002
Citrobacter freundii ATCC 8090	0–0.002
Proteus vulgaris ATCC 13315	0–0.002
Proteus mirabilis ATCC 7002	0–0.002
Shigella sonnei ATCC 25931	0–0.002
Pseudomonas aeruginosa ATCC 27853	0–0.002
Acinetobacter sp. strain ATCC 12606	0–0.002
Candida tropicalis	0–0.002

[a] Overnight cultures were used to prepare cell suspensions of approximately 10^8 cells per ml. Dilutions were tested for PnC in the EIA.

Quantitation of PnC levels in sputum. A total of 57 sputum samples from 47 patients were tested by the EIA. Of these, 20 grew *S. pneumoniae* (Table 2), and 18 of 20 (90%) were positive by EIA by using a cutoff point of >0.5 μg of PnC per ml. Of the 37 samples that did not grow *S. pneumoniae*, 3 were positive (overall specificity, 91.8%). Differences between the mean PnC level in sputum that yielded *S. pneumoniae* and sputum that yielded other bacteria were statistically significant ($P = 0.0001$).

The charts of all 47 patients were reviewed. Analysis of the diagnostic sensitivity and specificity of the EIA was

TABLE 2. PnC in sputum specimens of Alaska native patients with a suspected pneumonia caused by various bacterial pathogens[a]

Sputum culture report	No. tested	No. (%) EIA positive[b]
Streptococcus pneumoniae	20	18 (90)
Haemophilus influenzae	1	0 (0)
Pseudomonas sp.	3	0 (0)
Normal flora	20	2 (10)
No growth	2	0 (0)
Staphylococcus aureus	2	0 (0)
Staphylococcus sp.	2	0 (0)
Yeasts	1	0 (0)
Escherichia coli	1	0 (0)
Enterobacter sp.	1	0 (0)
Proteus sp.	1	0 (0)
Klebsiella pneumoniae	1	0 (0)
Morganella sp.	1	1 (100)
Unknown	1	0 (0)
Total non-*Streptococcus pneumoniae*	37	3 (8)

[a] A total of 57 patient sputum specimens were examined.
[b] PnC, >0.50 μg/ml.

restricted to those 34 patients from whom sputum specimens were collected within 24 h of the first radiographic report of a new pneumonia. Of these, cultures of blood from 18 patients were also performed; one of the blood cultures was positive for *S. pneumoniae*. Of the 34 patients with pneumonia, 12 produced sputum that grew *S. pneumoniae* as the reported pathogen. All 12 of these sputum samples had PnC levels of >0.5 μg/ml and were considered positive (sensitivity, 100%). PnC levels in the sputum of these patients ranged from 1.43 to 57.53 μg/ml (mean, 18.3 ± 19.4 μg/ml) (Fig. 1). The patient whose blood and sputum grew *S. pneumoniae* on culture had the highest level of PnC in sputum (57.53 μg/ml). There were 22 patients with pneumonia whose sputa did not grow *S. pneumoniae* on culture. Two of these samples were positive by EIA, with PnC levels of 2.70 and 4.53 μg/ml. The sputum of one patient grew a *Morganella* species, and the sputum of the other patient grew only normal flora. Examination of the antibiotic histories of both of these patients revealed that they both had been treated with antibiotics before sputum collection. The specificity and positive predictive value of the assay for detecting presumptive pneumococcal pneumonia were 90.1% (20 of 22 sputum samples) and 85.7% (12 of 24 sputum samples), respectively.

Smears of sputum samples from 25 patients with radiographic evidence of pneumonia were available for review. Of these, 19 were classified as "adequate," with polymorphonuclear leukocyte to epithelial cell ratios of ≥5. Of 10 adequate samples that grew *S. pneumoniae*, gram-positive diplococci were present in the smears of 9 samples (sensitivity, 90%) and were positive by EIA (sensitivity, 100%).

Of nine adequate samples that did not grow *S. pneumoniae*, gram-positive diplococci were present in the smears of three samples (specificity, 66%) and seven were EIA positive (sensitivity, 77%). The positive predictive values of the Gram stain and PnC EIA by using adequate samples were 75 and 83%, respectively. Of six inadequate samples (polymorphonuclear leukocyte/epithelial cell <5), the Gram stain contained organisms resembling pneumococci in two samples, one of which grew *S. pneumoniae* and was also positive by the EIA.

The EIA was positive for samples from 5 of 12 patients who did not have radiographic evidence of pneumonia. This included three patients whose sputa grew *S. pneumoniae*. The PnC levels in patients who did not have pneumonia, yet whose sputa grew *S. pneumoniae*, were 1.59, 3.03, and 18.75 μg/ml. Two of these three patients had radiographic chest abnormalities other than an infiltrate and may have had acute lower respiratory infections other than pneumonia. The mean PnC level in the sputa of these three patients was 7.70 ± 9.5 μg/ml; this could not be statistically distinguished from the mean PnC level of 18.30 μg/ml in the sputa of patients

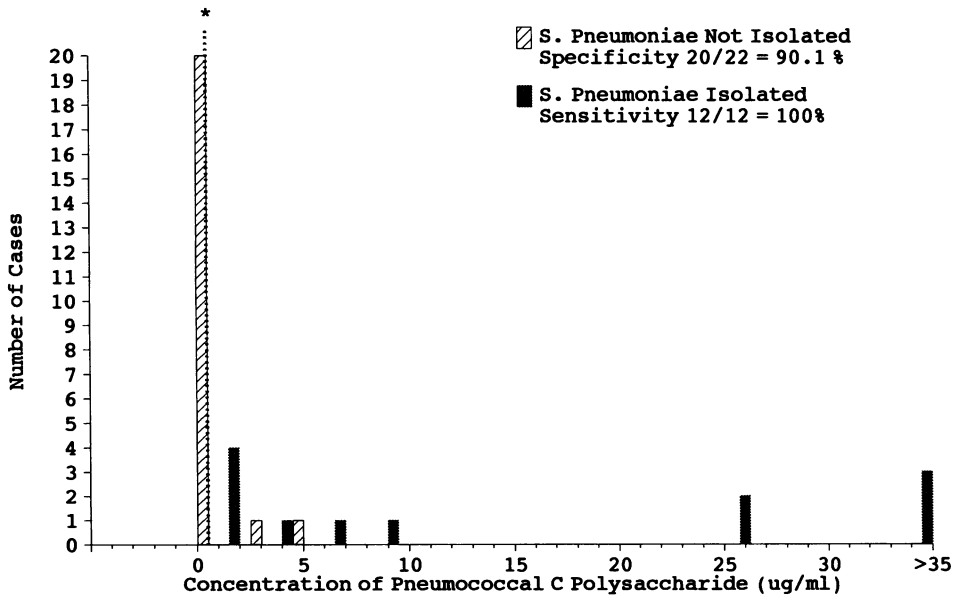

FIG. 1. Quantitation of PnC by EIA in the sputa of patients with radiographic documentation of pneumonia. *, cutoff of 0.50 μg of PnC per ml.

who had radiographic evidence of pneumonia and whose sputa grew *S. pneumoniae*.

DISCUSSION

Gram staining and culture of expectorated sputum remain the standard noninvasive methods for making a presumptive diagnosis of pneumococcal pneumonia. Because direct examination of sputum often reflects carriage of pneumococci in the respiratory tract, these methods remain relatively insensitive and nonspecific. Methods such as the evaluation of sputum quality (14), removal of respiratory tract contaminants by washing (2), and quantitative culture of liquefied sputum (6) may improve the sensitivity and specificity of sputum culture; however, these methods are not widely used by diagnostic microbiology laboratories. The quantitation of PnC levels in sputum by EIA provides a rapid and convenient method of processing large numbers of sputum samples for the presumptive diagnosis of pneumococcal pneumonia. The assay is sensitive and specific and clearly differentiates between those patients with pneumococcal pneumonia and those with pneumonia of other or unknown causes.

Of 22 patients who had pneumonia but whose sputa did not grow *S. pneumoniae*, sputa from only 2 of them were positive by the EIA. PnC levels in the sputa of both patients were <5.0 μg/ml, and both were pretreated with antibiotics before sputum collection, suggesting that PnC was present in the sputum but that there were no viable pneumococci. Although the start of antibiotic therapy rapidly decreases the number of viable pneumococci in sputum, it has been shown that pneumococcal capsular polysaccharide antigen can be readily detected for up to 5 days following the first isolation of pneumococci from sputum (19). However, unlike the pneumococcal capsular polysaccharide, PnC is associated with the cell wall and therefore may be sensitive to the action of β-lactam antibiotics. In our earlier study (15), we found that the detection of PnC in serial sputum samples from patients with presumptive pneumococcal pneumonia appeared to parallel the recovery of viable *S. pneumoniae* from sputum. This has been demonstrated by others (8) and suggests that PnC may not persist long after the beginning of antibiotic therapy. Therefore, PnC detection in sputum may be of limited value in patients who have been pretreated with antibiotics. On the other hand, quantitation of PnC levels in serial sputum samples may prove to be useful for monitoring antibiotic effectiveness in patients with pneumococcal pneumonia.

The detection of PnC in two patients whose sputa did not grow *S. pneumoniae* suggests, first, that the assay reagents may cross-react with other nonpneumococcal components present in the sample; second, it may represent difficulties associated with the laboratory identification of pneumococci in sputum samples. Immunologic cross-reactivity between *H. influenzae* and both polyclonal and monoclonal antibodies prepared against PnC or phosphocholine has been reported by others (7, 11, 18, 24).

In our study, only low levels of PnC (–0.02 μg/ml) were detected in standardized suspensions of other bacterial and yeast isolates. These levels were well below the 0.5-μg/ml cutoff point used for the detection of PnC in sputum samples. This suggests that significant cross-reactions may not be common by this assay and may also account for the greater specificity of this assay when compared with the results obtained by others (8, 11).

The presence of PnC in sputum without detectable *S. pneumoniae* organisms could be related to difficulties associated with the microbiologic recovery from sputum and the identification of this organism in sputum. These problems frequently include the overgrowth of the more fastidious pneumococci by morphologically similar flora, such as viridans group streptococci, that normally reside in the respiratory tract or the absence of viable pneumococci in aged sputum samples and possibly the presence of optochin-resistant or bile-insoluble atypical pneumococci (5). Detection of PnC in sputum may provide an additional parameter for the laboratory identification of pneumococci in sputum.

The potential ability of the quantitative EIA to distinguish between samples from patients with presumptive pneumococcal pneumonia from those patients with pneumococcal carriage was not evaluated in this study. However, the sputa of five patients admitted with a clinical suspicion of pneumonia, but who did not have radiographic confirmation of an infiltrate, were positive by EIA. *S. pneumoniae* grew in the sputa from three of these patients, suggesting pneumococcal carriage. While levels of PnC were low (<5.0 μg/ml) in two patients and high (18.75 μg/ml) in one other patient, these levels could not allow a distinction to be made from patients with presumptive pneumococcal pneumonia. Since patients with pneumonia caused by pathogens other than pneumococci may also carry *S. pneumoniae* in their oropharynxes, additional examination of sputum from patients with proven carriage is planned.

Of interest was the finding that of 18 patients whose blood was cultured, the blood and sputum from one patient grew *S. pneumoniae*. This patient also had the highest PnC level in sputum (57.53 μg/ml), suggesting that the detection of high levels of PnC in sputum may be a useful indicator of disease severity.

Other EIA systems have been proposed for the detection of PnC in the sputa of patients with pneumonia. Holmberg et al. (8) used a monoclonal antibody (immunoglobulin M)-based EIA for the detection of PnC in the sputa of patients with pneumonia. They reported a sensitivity and specificity of the EIA for the detection of presumptive pneumococcal pneumonia of 85 and 94.1%, respectively, using sputum samples collected on the day of admission. We recently evaluated (15) a commercial prototype assay using the same patients and samples described in this report. Major differences between the quantitative EIA described above and the commercial prototype assay were the monoclonal and polyclonal antibody reagents used, the assay format (in the prototype assay, the sample and secondary detector antibody were added to the assay simultaneously), and the cutoff point for determining a positive from a negative test results. In the prototype assay, the cutoff point was set at 0.25 optical density units, which corresponded to 0.2 μg/ml in the quantitative EIA. While both the quantitative EIA and the prototype EIA accurately detected PnC in sputum that was obtained from all 12 patients with pneumonia and that grew *S. pneumoniae*, the more conservative cutoff point of the quantitative EIA (0.5 μg/ml) gave fewer false-positive results and an improved specificity (90.1%) when it was compared with that of the commercial prototype EIA (specificity, 78.2%).

In conclusion, we developed an EIA for the quantitation of PnC in sputum. This assay is sensitive and specific for the diagnosis of presumptive pneumococcal pneumonia and possesses the potential for improving the accuracy of sputum examination for *S. pneumoniae*, monitoring the effectiveness of antibiotic therapy, monitoring disease severity, and perhaps, distinguishing between invasive pneumococcal disease and pneumococcal carriage.

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