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Detection of *Streptococcus pneumoniae* Antigen in Bronchoalveolar Lavage Fluid Samples by a Rapid Immunochromatographic Membrane Assay

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We conducted a retrospective study to evaluate an immunochromatographic membrane test (ICT), applied to bronchoalveolar lavage (BAL) fluid samples obtained in patients with suspected pneumonia, for the detection of *Streptococcus pneumoniae* antigen. The NOW *Streptococcus pneumoniae* test was assessed on 96 BAL fluid samples. Sensitivity was tested in 20 samples obtained from patients diagnosed as having pneumococcal pneumonia (growth of *S. pneumoniae* in blood cultures and/or in BAL fluid samples of $\geq 10^4$ CFU/ml). Specificity was tested in BAL fluid samples of nonpneumococcal etiology ($n = 41$) and in samples with no respiratory pathogen and a total bacterial count of $<10^4$ CFU/ml ($n = 35$). Using the ICT, pneumococcal antigen was detected in 29 (30.2%) BAL fluid samples, with a sensitivity of 95.0% (95% confidence interval [CI], 90.6% to 99.4%) and a specificity of 86.8% (95% CI, 80.1% to 93.8%). The ICT was easy to perform and revealed unequivocal and reproducible results. No interference was observed with high cell counts, red blood cells, or elevated protein levels. Four out of 10 false-positive readings occurred in samples with *S. pneumoniae* counts below the 10^4 CFU/ml threshold limit of pneumonia. In BAL fluid samples obtained after pneumococcal bacteremia, positive test results were found for up to 35 days after bacteremia. The ICT test applied to BAL fluid specimens is reproducible and accurate in the diagnosis of pneumococcal antigen. Further studies are required to establish the impact of the ICT on patient care.

Streptococcus pneumoniae prevails as a leading cause of community-acquired pneumonia (CAP), and it ranks as one of the organisms most commonly involved as the cause of severe CAP requiring intensive care admission (1, 9). Recently, an immunochromatographic membrane test (ICT) for rapid detection of *S. pneumoniae* antigen (NOW *Streptococcus pneumoniae* test; Binax, Portland, Maine) has been marketed. Till now, the test has been validated for urine and cerebrospinal fluid samples only. According to the package leaflet of the NOW *Streptococcus pneumoniae* test, the detection limit of the test in cerebrospinal fluid is 5.10^4 CFU/ml.

In our hospital, bronchoscopy with bronchoalveolar lavage (BAL) is used in the diagnosis of severe or nonresolving CAP, ventilator-associated pneumonia (VAP), and pneumonia in immunocompromised patients (7). As the BAL procedure dilutes the lung secretions 10- to 100-fold, a BAL fluid colony count of $\geq 10^4$ CFU/ml represents a bacterial load of $\geq 10^5$ to 10^6/ml at the collection site, which is indicative of pneumonia (2). As this 10^4 CFU/ml threshold is within reach of the ICT, we were interested in evaluating its performance characteristics when applied on BAL fluid samples obtained in patients with suspected pneumonia.

MATERIALS AND METHODS

Setting and samples. This retrospective study was conducted at the University Hospital of Maastricht, a 600-bed tertiary care hospital. Bronchoscopy with BAL was performed in patients with suspected pneumonia, including severe or nonresolving CAP, VAP, and pneumonia in immunocompromised patients. To test the sensitivity of the ICT, a group of BAL fluid samples obtained from patients diagnosed as having pneumococcal pneumonia was elected: diagnosis was made by microbiological culture, i.e., growth of *S. pneumoniae* in blood cultures drawn within 24 h before or after bronchoscopy and/or growth in BAL fluid samples in quantities of $\geq 10^4$ CFU/ml. Specificity and cross-reactions were tested in samples of patients with nonpneumococcal pneumonia, i.e., BAL fluid samples with a clear respiratory pathogen demonstrated (influenza virus, respiratory syncytial virus, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella pneumophila*) or with microorganisms other than *S. pneumoniae* in quantities of $\geq 10^4$ CFU/ml. Furthermore, a group of BAL fluid samples in which pneumonia was suspected but not microbiologically confirmed were included: in these samples, no respiratory pathogen was detected, and quantitative cultures did not reach the 10^4 CFU/ml threshold level. In some samples belonging to the latter two groups, *S. pneumoniae* was cultured, although in amounts less than 10^4 CFU/ml.

Sampling technique. A fiber-optic bronchoscope (Pentax FB-15H/FB-15X; Pentax Medicals, Tokyo, Japan) was introduced and "wedged" into the affected segmental or subsegmental bronchus. Four aliquots of 50 ml sterile saline (0.9% NaCl, room temperature) were instilled and immediately aspirated and recovered. The BAL fluid samples were transported to the laboratory within 15 min after collection and analyzed within 1 h upon arrival in the laboratory.

Laboratory processing. The volume of the BAL fluid was recorded. The first fraction, representing the bronchial fraction, was separated for mycobacterial culture, and the remaining fractions were pooled for further analysis. The total cell count was performed in a Fuchs Rosenthal hemocytometer chamber, and cytocentrifuge preparations were made as previously described. A differential cell count was made on cytocentrifuged preparations, and squamous epithelial cells were recorded as a percentage of a 500-nucleated-cell count (7).

All BAL fluid samples were quantitatively cultured for bacteria and yeasts on standard media using 2- and 10-μl volumes transferred by pipettes on appropriate media (8). BAL fluid samples were also cultured for filamentous fungi and mycobacteria and for *Legionella* spp. In addition, stains for detection of *Pneumocystis carinii* and filamentous fungi (methenamine-silver stain), *L. pneumophila* (immunofluorescent antibody stain), and acid-fast bacteria (auramine-rhodamine stain) were performed on cytocentrifuged preparations. Homemade PCRs for the detection of *C. pneumoniae* and *M. pneumoniae* were performed. For detection of *C. pneumoniae*, the primers used were 5′-TTA TTA ATT GAT

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GGT ACA ATA-3′ (corresponding to position 176,454 to 176,474 of the *C. pneumoniae* genome, which is part of the gene encoding the major outer membrane protein; GenBank accession number AE017159) and 5′-ATC TAC GGC AGT AGT ATA GTT-3′ (corresponding to position 176,660 to 176,640 of the genome). For detection of *M. pneumoniae* by PCR, the primers used were 5′-AAG GAC CTG CAA GGG TTC GT-3′ (corresponding to position 8,227 to 8,246 of the *M. pneumoniae* genome, which is part of the 16S rRNA gene; GenBank accession number AE000008) and 5′-CTC TAG CCA TTA CCT GCT AA-3′ (corresponding to position 8,503 to 8,484 of the genome). Viral cultures for herpes simplex virus, cytomegalovirus, adenovirus, influenza A-B, parainfluenza 1-3, and respiratory syncytial virus were done.

BAL fluid samples were centrifuged at 400 × *g* for 10 min, and the supernatant was stored at −70°C until ICT analysis.

BAL fluid samples were excluded if the retrieved volume was less than 20 ml, if the total cell count was less than 60,000/ml, or if the preparations showed excessive amounts of red blood cells, intercellular debris, or damaged red blood cells.

Binax NOW *Streptococcus pneumoniae* test (ICT). BAL fluid supernatant samples were thawed immediately before testing. The ICT was performed according to the instructions of the manufacturer for urine and cerebrospinal fluid specimens. Briefly, a swab was dipped into the samples to be tested and inserted into the test device consisting of a nitrocellulose membrane containing complexes of rabbit antibody against *S. pneumoniae* conjugated with colloidal gold particles. Next, three drops of citrate buffer solution were added and the device was closed, bringing the sample in contact with the test strip. After 15 min of incubation at room temperature, the presence of the control line (positive procedural control) and the clearing of the background in the result window (negative procedural control) were visually checked. The presence of a pink-to-purple colored sample line indicative of a positive result was recorded, and the intensity of this line was scored as clearly or weakly positive. According to the package leaflet, any visible line was interpreted as a positive result. If no sample line was observed, the assay was considered negative. Readings were performed by two observers, of whom the second was blinded. For each test kit, positive and negative control swabs provided by the manufacturer were tested.

Additional analysis. To assess reproducibility, a panel of samples with positive, weakly positive, and no sample lines was blindly retested. Samples with apparent false-positive readings were boiled for 5 min and centrifuged at 800 × *g* for 10 min and retested (16). Stability of the ICT readings after storage at −70°C was evaluated in two ICT-positive BAL fluid samples by storing multiple fractions at −70°C and testing a sample every 2 months for a period of 14 months.

In order to evaluate the persistence of *S. pneumoniae* antigen in BAL fluid samples after pneumonia, BAL fluid samples obtained ≥5 days after a bacteremic *S. pneumoniae* pneumonia (e.g., in cases of suspected VAP) were retrieved for ICT testing. In an attempt to explain unexpected results, patients' clinical records were reviewed.

RESULTS

A total of 96 BAL fluid samples were obtained from 93 patients (mean age of 54.0 ± 19.4 years, male-to-female ratio of 1.8:1). Sixty-five (69.9%) patients were hospitalized at the intensive care unit. The samples had been stored at −70°C for a median period of 36 (range, 0 to 84) months. Consecutive ICT testing of samples of the freshly stored BAL fluid samples remained positive for the period of 14 months tested.

Both observers unequivocally interpreted the presence or absence of the sample line. This sample line was clearly visible in 23 of 96 (24.0%) samples. Another six (6.2%) samples showed a weakly positive line. In the remaining 67 (69.8%) samples, no sample line was observed; these readings were considered negative. Ten samples with a clearly visible sample line, four samples with a weakly positive sample line, and eight samples with a negative reading were reassessed for reproducibility and yielded results identical to those obtained at the first test run. No apparent interference was observed in samples that were macroscopically bloody ($n = 30$) nor in samples with high total cell counts ($\geq 10^6$ cells/ml, $n = 44$) or high protein contents (≥500 mg/liter, $n = 30$).

Table 1 lists the readings of the ICT in BAL fluid samples related to the corresponding culture results. The ICT yielded positive results in 19 of 20 samples of pneumococcal pneumonia, resulting in a sensitivity of 95.0% (95% confidence interval [CI], 90.6% to 99.4%). In the nonpneumococcal pneumonia and in the "pneumonia not confirmed" group combined ($n = 76$), the test showed 10 false-positive readings, which equals a specificity of 86.8% (95% CI, 80.1% to 93.8%).

In four patients with pneumococcal bacteremia, *S. pneumoniae* did not grow at all in three BAL fluid samples and only at a count of 10^2 CFU/ml in another sample. In three of these samples, the ICT test was positive. Upon review of the clinical record, the BAL fluid sample with the false-negative ICT appeared to be obtained from a patient with *S. pneumoniae* mitral valve endocarditis, and the radiographic infiltrates were considered to be consistent with pulmonary edema.

All 10 apparent false-positive samples gave identical results when retested after boiling. Half of them showed a weakly positive sample line. The patient for whom *C. pneumoniae* was recovered from the BAL fluid had blood cultures grown with *S. pneumoniae* 3 weeks before. From one false-positive sample, the *Staphylococcus aureus*, *Haemophilus influenzae*, and group B streptococcus strains were still available: boiled solutions of these strains were ICT tested but did not show positive readings. From eight patients, clinical data were available; seven of them were receiving antibiotics active against *S. pneumoniae* prior to or at the day of bronchoscopy.

In the present collection, three samples had ≥1% squamous epithelial cells, including two samples of the "no pneumonia group," with negative ICT readings. A third sample (with 2.4% squamous epithelial cells) was categorized in the pneumococcal pneumoniae group. The differential cell count of the latter sample showed 95.2% polymorphonuclear neutrophils with 15.6% intracellular organisms, presenting as diplococci with a faint and decolorized appearance.

Persistence of *S. pneumoniae* antigen was studied in 20 BAL fluid samples; the ICT remained positive in 11 of 15 BAL fluid samples obtained within 25 days after bacteremic pneumonia and was positive for up to 35 days (Table 2).

DISCUSSION

The present study validated the technical applicability of the NOW *Streptococcus pneumoniae* ICT test applied on BAL fluid specimens and demonstrated an excellent sensitivity and a good specificity for the diagnosis of pneumococcal pneumonia.

There are several limitations in the study. Its retrospective design did not allow paired comparison with other diagnostic methods, in particular the ICT performed on urine samples. In addition, it did not allow us to consider issues of prevalence and predictive values nor to assess its diagnostic value against clinical and radiographic criteria of pneumonia. Furthermore, the accuracy of the 10^4 CFU/ml threshold level of quantitative culture is not absolute, and overlaps by 1 log at both sites of the threshold are possible (2, 8).

The reported sensitivities of the ICT applied on urine samples range from 44.2% to 88.8% (4, 5, 12, 13, 14, 17). Sensitivities are higher in bacteremic versus nonbacteremic patients and increase when urine samples are concentrated, which is mostly performed by ultracentrifugation and may last for 1 to

TABLE 1. Results of the Binax NOW *Streptococcus pneumoniae* ICT for the detection of *S. pneumoniae* in BAL fluid samples

Patient clinical status	No. of BAL fluid samples	No. of positive ICT results (%)
Pneumococcal pneumonia diagnosed by microbiological culture[a]		
Blood *S. pneumoniae* and BAL fluid $\geq 10^4$ CFU/ml *S. pneumoniae*	3	3
Blood culture no growth, BAL fluid $\geq 10^4$ CFU/ml *S. pneumoniae*	7	7[b]
No blood cultures drawn, BAL fluid $\geq 10^4$ CFU/ml *S. pneumoniae*	6	6[b]
Blood *S. pneumoniae* and BAL fluid 1.10^2 CFU/ml *S. pneumoniae*	1	1
Blood *S. pneumoniae* and BAL fluid no growth	3	2
Total	20	19 (95)
Nonpneumococcal pneumonia		
Other respiratory pathogen detected or BAL fluid culture $\geq 10^4$ CFU/ml, blood culture no growth of *S. pneumoniae*		
Chlamydia pneumoniae	1	1[b]
Legionella pneumophila	2	0
Mycoplasma pneumoniae	3	0
Influenza A virus	2	0
Respiratory syncytial virus	4	0
Group A hemolytic streptococcus $\geq 10^4$ CFU/ml	1	0
Staphylococcus aureus $\geq 10^4$ CFU/ml	6	1[b]
Streptococcus anginosus $\geq 10^4$ CFU/ml	2	0
Streptococcus viridans $\geq 10^4$ CFU/ml	1	0
Enteric gram-negative rods $\geq 10^4$ CFU/ml	4	0
Pseudomonas aeruginosa $\geq 10^4$ CFU/ml	9	0
Staphylococcus aureus, *H. influenzae*, group B streptococcus $>10^4$ CFU/ml	1	1
Other respiratory pathogen $\geq 10^4$ CFU/ml with *S. pneumoniae* $<10^4$ CFU/ml, blood cultures no growth of *S. pneumoniae*		
H. influenzae $\geq 10^4$ CFU/ml + *S. pneumoniae* 2.5×10^2 CFU/ml	1	0
Staphylococcus aureus $\geq 10^4$ CFU/ml + *S. pneumoniae* 2.5×10^3 CFU/ml	1	0
H. influenzae $\geq 10^4$ CFU/ml + *S. pneumoniae* 2.10^3 CFU/ml	1	0
H. influenzae $\geq 10^4$ CFU/ml + *S. pneumoniae* 5.10^3 CFU/ml	2	1
Total	41	4 (9.8)
Pneumonia microbiologically not confirmed		
No respiratory pathogen detected and BAL fluid culture $<10^4$ CFU/ml, blood cultures no growth of *S. pneumoniae*		
No growth of bacteria	14	1
Oral flora $<10^4$ CFU/ml	15	1[b]
Growth of *S. pneumoniae* $<10^4$ CFU/ml, blood cultures no growth of *S. pneumoniae*		
S. pneumoniae 3.10^2 CFU/ml	1	0
S. pneumoniae 1.10^3 CFU/ml	1	1
S. pneumoniae 2.10^3 CFU/ml	1	1[b]
Staphylococcus aureus 1.10^3 CFU/ml + *S. pneumoniae* 1.10^3 CFU/ml	1	0
H. influenzae 1.10^3 CFU/ml + *S. pneumoniae* 1.10^3 CFU/ml	1	1[b]
H. influenzae 1.10^3 CFU/ml, *Staphylococcus aureus* 1.10^3 CFU/ml + *S. pneumoniae* 2.10^3 CFU/ml	1	1
Total	35	6 (17.1)

[a] *S. pneumoniae* was part of a mixed flora in 9 of 20 samples. The copathogens included influenza A virus, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, group C hemolytic streptococci, *H. influenzae*, *Escherichia coli*, and *Staphylococcus aureus* (2×); all bacteria were recovered in quantities of $\geq 10^4$ CFU/ml.
[b] Weakly positive results.

4 h (12, 13, 14). Specificity of the ICT urine test has been reported to be low in children (due to nasopharyngeal carriage) (6), and adult patients showed a specificity of 89.7% to 100%, depending on the standard of comparison (4, 12, 14). The sensitivity of the ICT in BAL fluid samples in the present study was excellent. The single false-negative result occurred in a patient with pulmonary edema and *S. pneumoniae* endocarditis, and this sample was, in retrospect, erroneously assigned to the "pneumococcal pneumonia" group. Part of the false-positive readings occurred in samples with *S. pneumoniae* at counts below the 10^4 CFU/ml threshold, and half of them showed a weakly positive sample line, which represents, according to the package leaflet, low antigen levels. As these samples did not display elevated numbers of squamous epithelial cells, oropharyngeal contamination (with detection of pneumococcal colonization rather than infection) is less likely. Because most of these apparent false-positive samples were obtained in patients on antibiotics, we assume that they may

TABLE 2. Persistence of *S. pneumoniae* antigen as detected by the ICT in BAL fluid samples obtained in patients after recovery from bacteremic pneumococcal pneumonia

Delay of bronchoscopy after initial bacteremia	ICT result	
	Positive (*n*)	Negative (*n*)
≥5 to 10 days	3	1
≥10 to 15 days	3[a]	1
≥15 to 20 days	2[a]	0
≥20 to 25 days	3	2
Day 26	0	1
Day 35	1	0
Days 65, 198, 236	0	3

[a] Including one weakly positive result.

represent episodes of pneumococcal pneumonia in which cultures failed to reach the 10^4 CFU/ml level. Finally, another false-positive result was obtained in a patient with a bacteremic *S. pneumoniae* pneumonia 3 weeks earlier.

What is the place of the ICT performed on BAL fluid samples? In our collection of BAL fluid samples over the period considered, *S. pneumoniae* in quantities of $\geq 10^4$ CFU/ml accounted for only 23 of 1,042 (2.2%) of all samples submitted for culture, which means that indiscriminate use of the ICT on BAL fluid would result in only a 16% positive predictive value, precluding its clinical use. In line with others, we reserve bronchoscopy for CAP in immunocompromised or human immunodeficiency virus-infected patients, in patients with a fulminant course, and in those requiring admission to the intensive care and having unresolving pneumonia. In these cases, cytological and microbiological diagnostic work-up of the BAL fluid can detect a number of infectious and noninfectious causes (1, 11). In addition, by the time of bronchoscopy most if not all patients are on antibiotics, which are known to damage Gram stain reaction and morphology and hamper culture (3). In samples with high percentages of polymorphonuclear neutrophils and nonstained or damaged or faintly stained bacteria seen on Gram staining, we perform the ICT as an adjunct to standard cytological and microbiological analysis. Using the ICT on BAL fluid specimens may also be useful when samples have been exposed to a transportation delay that is too long or too cold. The long shelf life (1 year for a package of 12 tests) and its stability at room temperature allow incidental use. In cases of a positive reading, the possibility of a mixed flora or a superinfecting pathogen should always be considered, as illustrated by the number of copathogens recovered in the pneumococcal pneumonia group in the present study and as mentioned by others (10). Further, the ICT on BAL fluid may be used as an investigational tool in epidemiological studies that assess BAL fluid samples in patients with pneumonia of unknown origin, of which *S. pneumoniae* is probably the leading cause (15). In that way, the persistence of the antigen may be advantageous, and at present we are considering a retrospective study on BAL fluid specimens obtained from patients within 3 days after their admission to the hospital. However, with regard to the ICT sensitivity, a prospective study with a side-by-side comparison of the ICT performed on urine and BAL fluid samples is needed to demonstrate possible differences in test characteristics.

In conclusion, we validated the technical applicability of the Binax NOW *Streptococcus pneumoniae* antigen test applied to BAL fluid specimens in the diagnosis of pneumococcal pneumonia. The ICT applied on BAL fluid specimens may be useful in selected cases and in epidemiological studies.

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