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Evaluation of a multiplex PCR for bacterial pathogens applied to bronchoalveolar lavage

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ABSTRACT: The present study assessed the diagnostic usefulness of a multiplex PCR (mPCR) for *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* applied to bronchoalveolar lavage (BAL).

Fibreoptic bronchoscopy was performed on 156 hospitalised adult patients with lower respiratory tract infection (LRTI) and 36 controls. BAL fluid was analysed with bacterial culture and mPCR.

By conventional diagnostic methods, *S. pneumoniae*, *H. influenzae*, *M. pneumoniae* and *C. pneumoniae* were aetiological agents in 14, 21, 3.2 and 0% of the LRTI patients, respectively. These pathogens were identified by BAL mPCR in 28, 47, 3.2 and 0.6% of cases, respectively, yielding sensitivities of 86% for *S. pneumoniae*, 88% for *H. influenzae*, 100% for *M. pneumoniae* and 0% for *C. pneumoniae*, and specificities of 81, 64, 100 and 99% for *S. pneumoniae*, *H. influenzae*, *M. pneumoniae* and *C. pneumoniae*, respectively. Of the 103 patients who had taken antibiotics prior to bronchoscopy, *S. pneumoniae* was identified by culture in 2.9% and by mPCR in 31%. Among the controls, mPCR identified *S. pneumoniae* in 11% and *H. influenzae* in 39%.

In lower respiratory tract infection patients, bronchoalveolar lavage multiplex PCR can be useful for identification of *Streptococcus pneumoniae*, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*. The method appears to be particularly useful in patients treated with antibiotics.

KEYWORDS: Bronchoalveolar lavage, *Haemophilus influenzae*, lower respiratory tract infection, *Mycoplasma pneumoniae*, PCR, *Streptococcus pneumoniae*

In cases of severe community-acquired lower respiratory tract infection (LRTI), it is desirable to identify the pathogen causing the infection in order to choose appropriate antibiotic treatment. Cultures from the lower respiratory tract are often used because blood cultures have low sensitivity for LRTI aetiology [1]. As sputum cultures may be contaminated by the oropharyngeal flora, so fibreoptic bronchoscopic techniques have been developed to enable collection of lower respiratory tract samples with minimal risk of contamination. At present, a major reason for fibreoptic bronchoscopy (FOB) in LRTI patients is failure to respond as expected to the first-line antibiotic treatment [2, 3]. However, PRATS *et al.* [4] demonstrated that the culture yield of *Streptococcus pneumoniae* and *Haemophilus influenzae* is rapidly reduced in lower respiratory tract secretions during antibiotic treatment; because of this, PRATS *et al.* [4] and other groups [5, 6] have suggested that FOB should be performed earlier in the course of LRTI, if possible prior to antibiotic treatment.

In contrast to culture, PCR is not dependent on viable bacteria. In a study by WHEELER *et al.* [7], PCR for *S. pneumoniae* often remained positive in sputum during antibiotic treatment, while sputum culture quickly became negative. Consequently, PCR applied to bronchoscopic samples may be useful for identification of *S. pneumoniae* and *H. influenzae* during antibiotic treatment. To the present author's knowledge, this has not been evaluated.

One reason for nonresponse to β-lactam antibiotics in LRTI is atypical pathogens as a cause of the infection. While PCR for *Legionella* spp. applied to bronchoalveolar lavage (BAL) fluid has been found useful [8], PCR for *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* has not been evaluated in BAL samples. However, as sputum samples have been shown to be more useful than upper respiratory tract samples for PCR identification of *M. pneumoniae* [9, 10] and *C. pneumoniae* [11], and because sputum samples can often not be collected in patients with LRTI,

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BAL PCR could probably be useful for identification of these two pathogens in LRTI patients.

The current authors have developed a multiplex PCR (mPCR) for simultaneous identification of *S. pneumoniae*, *H. influenzae*, *M. pneumoniae* and *C. pneumoniae* in respiratory tract samples. The method showed an analytical sensitivity of 100% (89 out of 89) and a specificity of 99% (167 out of 168) when 257 bacterial strains (37 different species) were tested [12]. mPCR has been evaluated on sputum samples and nasopharyngeal samples from adults with community-acquired pneumonia (CAP) and controls with promising results [13].

The aim of the present study was to estimate the diagnostic accuracy of mPCR applied to BAL samples from adult patients with LRTI. For further determination of the specificity of BAL mPCR, a control group including patients investigated on suspicion of malignancy was included.

MATERIALS AND METHODS

Study subjects

The present study was performed prospectively at the Silkeborg County Hospital, Silkeborg, Denmark, in its Dept of Internal Medicine, which has 100 beds. Silkeborg County Hospital is the only hospital in the region and serves a basic population of 100,000 inhabitants in the area. As previously described [14], all immunocompetent adult patients hospitalised for LRTI during weekdays between September 1997 and August 2000 were consecutively included in the present study. Thus, the included patients were unselected adult patients with LRTI requiring hospitalisation. The clinical diagnosis of LRTI required that the patient had fever (rectal temperature $\geqslant$37.6°C within 48 h of inclusion in the study) and/or an increased leukocyte count ($\geqslant 11 \times 10^9$ leukocytes·L^{-1}) in peripheral blood on admission, together with increased focal symptoms from the lower airways with at least one of three newly developed symptoms of increased dyspnoea, increased coughing and/or increased sputum purulence. Patients with known malignancy and patients with oxygen saturation <85% with a maximum of 1 L nasal oxygen were not included. Chest radiograph and spirometry were performed. Demographic, comorbidity and smoking data were collected, and information on prior antibiotic treatment for any diagnosis was obtained from either the individual patient or the patient's general practitioner.

Adult patients who consecutively underwent FOB for suspected malignancy were enrolled as controls. Information about prior antibiotic treatment and smoking data were collected.

Bronchoscopy

All patients and controls underwent a standardised FOB with BAL [15] within 24 h of admission. Prior to the FOB, the mouth and pharynx were anaesthetised with a solution of 4% lidocaine. The fibre bronchoscope was introduced through the nose or through the mouth and additional anaesthetics (1% lidocaine solution) were applied as the scope passed the larynx and trachea. A sterile, thin tube was introduced in the working channel of the bronchoscope. The tip of the scope was wedged in an appropriate segment in the bronchus, and thereafter lavage was performed. A segment of bronchus affected by a new pulmonary infiltrate, as seen at a prior chest radiograph, was chosen for lavage. In the LRTI patients without radiographically observed infiltrates and the control patients, the middle lobe was chosen for lavage. Between one and three portions of 60 mL of isotonic sodium chloride solution were used for lavage and the aspirated fluid was collected in one single portion for microbiological analyses.

Conventional microbiological investigations

BAL fluid and sputum samples from the LRTI patients and BAL fluid from the controls were analysed with culture at the Dept of Clinical Microbiology, Aarhus University Hospital (Aalborg, Denmark), within a maximum of 6 h from the time of sampling. The specimens were cultured on 5% horse blood agar and chocolate agar with semiquantitative determinations by dispersion of 1 and 10 μL on each half of the plate. The plates were incubated in 5% carbon dioxide at 35°C for 24–48 h. Bacterial identification was performed according to standard microbiological methods [16]. The cut-off limit for a positive BAL or sputum culture result was 10^2 colony-forming units (cfu)·mL^{-1} sample. After culture, the BAL fluid was frozen at -20°C.

BAL samples and throat swabs, which were placed in two sucrose phosphate chlamydial transport media, from the LRTI patients where sent to the Dept of Bacteriology, Mycology and Parasitology, Statens Serum Institute (Copenhagen, Denmark), and were analysed with singleplex PCR (sPCR) assays for *M. pneumoniae*, *C. pneumoniae*, *Chlamydophila psittaci*, and *Legionella* spp. [17, 18]. In addition, the BAL samples and throat swabs were cultured for *C. pneumoniae* [19].

From the LRTI patients, blood samples were collected for culture with a Bactec blood-culturing system (BioMérieux, Marcy-lEtoile, France) at the Dept of Clinical Microbiology, Aarhus University Hospital. Nonfrozen urine samples were sent to the Dept of Bacteriology, Mycology and Parasitology, Statens Serum Institute, and were analysed for pneumococcal capsular polysaccharides by countercurrent immunoelectrophoresis [20], and for *Legionella pneumophila* serogroup 1 antigen by enzyme immunoassay (Biotest AG, Dreieich, Germany).

Multiplex PCR

Frozen BAL samples from the LRTI patients and controls were sent to the Dept of Clinical Microbiology, Örebro University Hospital (Örebro, Sweden), for mPCR. A detailed description of the development and procedures of the mPCR is presented in a previous paper [12]. mPCR was performed under blinded conditions for simultaneous identification of specific genes for *S. pneumoniae* (*lytA*, 229 bp), *M. pneumoniae* (*P1*, 483 bp), *C. pneumoniae* (*ompA*, 368 bp), and *H. influenzae* (16S rRNA, 538 bp). The following primers were used: *lytA*, 5′-CGGACTACCGCCTTTATATCG-3′ and 5′-GTTTCAATC-GTCAAGCCGTT-3′; *P1*, 5′-ACTCGGAGGACAATGGTCAG-3′ and 5′-CAAACCCGGTCTTTTCGTTA-3′; *ompA*, 5′-ACACGA-TGCAGAGTGGTTCA-3′ and 5′-TGTTTACAGAGAATTGCGA-TACG-3′; and 16S rRNA, 5′-TCCTAAGAAGAGCTCAGAGAT-3′ and 5′-TGATCCAACCGCAGGTTCC-3′. The concentration of all primers was 50 μM. Briefly, DNA from 0.2–0.5 mL BAL fluid was extracted using the automatic MagNa Pure LC DNA-Isolation system (Roche Diagnostics, Mannheim, Germany).

Extracted DNA (10 μL) was added to a 40-μL mixture to give a total mPCR mixture volume of 50 μL, which included 90 nM of each of the two *lytA* primers and 250 nM of each of the other six primers, 800 μM of the deoxyribonucleoside triphosphate mixture (Perkin-Elmer Applied Biosystem, Norwalk, CT, USA), 1.5 U of AmpliTaq Gold DNA Polymerase (Perkin-Elmer Applied Biosystem), and 10× PCR buffer (Perkin-Elmer Applied Biosystem). PCR amplification was performed on a GenAmp PCR system 9600 (Perkin-Elmer Applied Biosystem) with the following parameters: 94°C for 10 min followed by 40 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, with the last cycle concluding with 72°C for 7 min, before storage at 4°C. The amplified sample was examined by electrophoresis on an agarose gel containing ethidium bromide. The result was compared with a DNA molecular weight marker. In every PCR run, one negative control and positive controls for each one of the four investigated bacteria were included. Samples positive for the 16S rRNA gene were considered positive for *H. influenzae* if they were also positive in a verification PCR for the *P6* gene, using the same PCR protocol. The *P6* PCR mixture included 125 nM of each *P6* primer (5′-TTGGC-GGWTACTCTGTTGCT-3′ and 5′-TGCAGGTTTTTCTTCAC-CGT-3′). All mPCR-negative samples were spiked with a reference strain of *S. pneumoniae* and were re-run by the PCR protocol for control of inhibition.

By serial dilution of bacterial strains, the detection levels of the mPCR have been shown to be 10^2 cfu·mL^{-1} of sample for *S. pneumoniae* and *H. influenzae*, and 10^3 genome copies or inclusion-forming units per millilitre of sample for *M. pneumoniae* and *C. pneumoniae* [12].

Criteria for aetiologies and exclusion of aetiologies

The criteria for LRTI aetiologies used in the present study were as follows. *S. pneumoniae*: positive blood culture, positive urinary antigen test, positive BAL culture, or positive sputum culture; *H. influenzae*: positive blood culture, positive BAL culture, or positive sputum culture; *M. pneumoniae*: positive BAL or throat swab sPCR; *C. pneumoniae*: positive BAL or throat swab sPCR, or positive BAL or throat swab culture. These criteria were used in the calculations of sensitivities and specificities of BAL mPCR. If no available reference analysis was positive for a pathogen, the pathogen was ruled out as an aetiological agent in the specificity analysis.

Ethics

The study was performed according to the Declaration of Helsinki II and was approved by the local ethical committee; all participating patients gave written consent.

RESULTS

Patients and controls

During the study period, 167 patients fulfilled the study criteria for LRTI. Eight patients declined participation, thus 159 patients were included in the prospective study. BAL fluid from 156 patients was available for mPCR and these patients were included in the present study. The LRTI patients had a median age of 63 yrs (range 26–90 yrs), 48 (31%) patients were current and 62 (40%) were previous smokers, and the mean pack-yrs history in the current and previous smokers was 27 yrs. A chronic lung disease was documented in 72 (46%) patients, and the mean forced expiratory volume in one second on day 7 from inclusion was 58% of predicted. New infiltrates were identified by radiography in 87 (56%) LRTI patients. The median interval between illness onset and FOB was 8 days; FOB was performed within 2 weeks of illness onset in 72% of the patients, and FOB was performed within 24 h of hospitalisation in all patients. In 103 (66%) patients, antibiotics had been taken within 7 days prior to FOB. The median length of hospital stay was 8 days (range 1–40 days). One patient died within 30 days of admission.

Out of the 51 controls included, 12 were excluded as they were diagnosed by radiography as having pulmonary infection with infiltrates (n=9), lung abscess (n=1), empyema (n=1), and bronchiectasies (n=1). One control was excluded as no BAL culture was performed, and another two controls were excluded as they had taken antibiotics during the preceding 7 days. Of the remaining 36 controls, 22 had lung malignancies and 14 had no pathology identified by FOB or radiological examinations. The median age of the 36 controls was 63 yrs (range 30–77 yrs), and 32 (89%) were current or previous smokers.

Identification of pathogens in patients with LRTI

In 55 LRTI patients (35%), any of the four studied pathogens was considered to be the aetiology, *i.e. S. pneumoniae* in 18 (12%) patients, *H. influenzae* in 30 (19%) patients, both *S. pneumoniae* and *H. influenzae* in two (1.3%) patients, *M. pneumoniae* in five (3.2%) patients, and *C. pneumoniae* in no patients.

S. pneumoniae was identified by blood culture in six (3.9%) out of 152 patients tested, by urinary antigen test in nine (6.3%) out of 142, by BAL culture in 10 (6.4%) out of 156, and by sputum culture in three (5.3%) out of 56. Two of those with positive sputum culture were also BAL-culture positive for *S. pneumoniae*. *H. influenzae* was identified by BAL culture in 31 (20%) out of 156 patients tested and by sputum culture in six (11%) out of 56, but in no blood culture. Five out of the six patients with positive sputum culture were also BAL-culture positive for *H. influenzae*. *M. pneumoniae* was identified in five patients by both BAL sPCR and throat swab sPCR. BAL sPCR was performed in 154 patients and throat swab sPCR in 156 patients. No patient was positive for *C. pneumoniae*, although BAL sPCR was performed in 155 patients, throat swab sPCR in 155, BAL culture in 154, and throat swab cultures in 154.

No patient was positive for *Legionella* spp. by urinary antigen test (154 tested), BAL sPCR (155 tested), or throat swab sPCR (156 tested). No patient was blood- or sputum-culture positive for *Staphylococcus aureus* or Gram-negative enteric bacilli. However, BAL culture identified *S. aureus* in 10 patients, Gram-negative enteric bacilli in 10 patients, *Moraxella* spp. in five patients, *Neisseria meningitidis* in three patients, and *Enterococcus faecalis* in one patient. sPCR identified *C. psittaci* in one patient.

Streptococcus pneumoniae

S. pneumoniae was an aetiological agent in 22 (14%) patients by the conventional tests, and was identified by BAL mPCR in 44 (28%) of the 156 LRTI patients. With all tests included, *S. pneumoniae* was identified in 30% of the LRTI patients.

TABLE 1 Performance of multiplex PCR (mPCR) applied to bronchoalveolar lavage (BAL) in 156 adults with lower respiratory tract infection

Species	Reference tests	Sensitivity[#]	Specificity[¶]	PPV[+]	NPV[§]
Streptococcus pneumoniae	Blood culture, BAL culture, sputum culture and urinary antigen test	86 (19/22)	81 (109/134)	43 (19/44)	97 (109/112)
Haemophilus influenzae	Blood culture, BAL culture and sputum culture	88 (28/32)	64 (79/124)	38 (28/73)	95 (79/83)
Mycoplasma pneumoniae	BAL PCR and throat swab PCR	100 (5/5)	100 (151/151)	100 (5/5)	100 (151/151)
Chlamydophila pneumoniae	BAL PCR, BAL culture, throat swab PCR and throat swab culture	0 (0/0)	99 (155/156)	0 (0/1)	100 (155/155)

PPV: positive predictive value; NPV: negative predictive value.[#]: Reported as percentage (number with positive mPCR/ number with any reference test positive); [¶]: reported as percentage (number with negative mPCR/ number with all performed reference tests negative); [+]: reported as percentage (number with any reference test positive/ number with positive mPCR); [§]: reported as percentage (number with all performed reference tests negative/ number with negative mPCR).

Table 1 demonstrates moderate-to-high sensitivity and negative predictive value, but low specificity and positive predictive value of BAL mPCR for *S. pneumoniae*. Among the six patients with pneumococcal bacteraemia, *S. pneumoniae* was identified by BAL mPCR in all six patients and by BAL culture in one patient. Likewise, of the nine patients with positive urinary antigen test, *S. pneumoniae* was identified in seven by BAL mPCR and in none by BAL culture. Among the 87 patients with infiltrates shown by radiography, mPCR for *S. pneumoniae* had a sensitivity of 94% (16 out of 17) and a specificity of 73% (51 out of 70).

Haemophilus influenzae

H. influenzae was an aetiological agent in 32 (21%) patients by the conventional tests, and was identified by BAL mPCR in 73 (47%) of the 156 LRTI patients. While the sensitivity and negative predictive value of BAL mPCR for *H. influenzae* were moderate to high, the specificity and positive predictive value were low (table 1). Among the 87 patients with infiltrates shown by radiography, mPCR for *H. influenzae* had a sensitivity of 84% (16 out of 19) and a specificity of 62% (42 out of 68).

H. influenzae was identified in 20% by BAL culture and 51% by BAL mPCR in the 110 current or previous smokers compared with 22% by BAL culture and 37% by BAL mPCR (not significant) in the 27 patients who had never smoked.

Mycoplasma pneumoniae

M. pneumoniae was an aetiological agent in five (3.2%) LRTI patients. There was total agreement between BAL sPCR, throat swab sPCR and BAL mPCR for identification of *M. pneumoniae* in the present study (table 1). None of these patients had any other pathogen identified.

Chlamydophila pneumoniae

No patient had positive sPCR or positive culture for *C. pneumoniae*. The patient with BAL mPCR positive for *C. pneumoniae* had no throat swab sPCR for *C. pneumoniae* performed, but BAL sPCR and the two *C. pneumoniae* cultures were negative.

Identification of more than one pathogen

The relationship between the different tests for *S. pneumoniae* and *H. influenzae* is shown in table 2. Both of these pathogens were identified in 18 patients as follows: *S. pneumoniae*, but not *H. influenzae*, was identified in 29 patients; and *H. influenzae*, but not *S. pneumoniae*, was identified in 59 patients. Among the 22 patients with *S. pneumoniae* aetiology, BAL culture was positive for *S. aureus* in two patients and *Moraxella* spp. in one patient. Two patients with *H. influenzae* aetiology were also BAL-culture positive for Gram-negative enteric bacilli.

Diagnostic influence of antibiotic treatment prior to bronchoscopy

Among 53 patients with no antibiotics taken prior to FOB, BAL culture and BAL mPCR identified *S. pneumoniae* in 13% (n=7) and 23% (n=12), respectively, and *H. influenzae* in 19% (n=10) and 42% (n=22), respectively, of the patients (nonsignificant). A bacterial concentration of $\geqslant 10^4$ cfu·mL^{-1} in BAL culture was identified in six out of seven patients identified with *S. pneumoniae* and in eight out of 10 patients identified with *H. influenzae*.

In the 103 patients with antibiotics taken prior to FOB, BAL culture and BAL mPCR identified *S. pneumoniae* in 2.9% (n=3) and 31% (n=32), respectively ($p<0.001$, Chi-squared test), and *H. influenzae* in 20% (n=21) and 50% (n=51), respectively (nonsignificant), of the patients. A bacterial concentration of $\geqslant 10^4$ cfu·mL^{-1} in BAL culture was identified in one out of three patients identified with *S. pneumoniae* and in 16 out of 21 patients identified with *H. influenzae*. One LRTI patient with pneumococcal bacteraemia had taken antibiotics prior to blood sampling and FOB, and *S. pneumoniae* was identified by BAL mPCR but not by BAL culture in that patient. Of the five patients with *M. pneumoniae* aetiology, three had taken antibiotics prior to FOB.

Bacterial identification in the control group

Table 3 shows the pathogens identified by BAL culture and BAL mPCR in the control group. Among the 36 controls, either BAL culture or BAL mPCR identified *S. pneumoniae* in six (17%) controls and *H. influenzae* in 14 (39%) controls. In the controls with lung malignancy, *S. aureus* was identified in three controls and Gram-negative enteric bacilli in two controls. *S. aureus* was also identified in one control without identified pathology. No control was mPCR positive for either *M. pneumoniae* or *C. pneumoniae*.

PCR inhibition

No PCR inhibition was identified in any BAL mPCR analysis.

TABLE 2 Combined results of tests for *Streptococcus pneumoniae* and *Haemophilus influenzae* in 156 patients with lower respiratory tract infection

Results regarding *S. pneumoniae*			Results regarding *H. influenzae*		Patients with the indicated result combination noted[##]
Blood culture and/or urinary antigen test[#]	BAL culture and/or sputum culture[¶]	BAL mPCR[+]	BAL culture and/or sputum culture[§]	BAL mPCR[f]	
+	-	+	-	-	6
-	+	+	-	-	8
-	+	-	-	-	1
-	-	+	-	-	14
+	+	+	-	+	1
+	-	+	+	+	1
+	-	+	-	+	2
+	-	-	+	+	1
+	-	-	-	+	1
-	+	+	-	+	1
-	-	+	+	+	3
-	-	+	+	-	2
-	-	+	-	+	6
-	-	-	+	+	23
-	-	-	+	-	2
-	-	-	-	+	34
-	-	-	-	-	49
No test performed	-	-	-	-	1

BAL: bronchoalveolar lavage; mPCR: multiplex PCR; +: positive; -: negative. [#]: positive, n=12; [¶]: positive, n=11; [+]: positive, n=44; [§]: positive, n=32; [f]: positive, n=73; [##]: the number of patients with the certain combination of results of the first five columns.

DISCUSSION

In pneumonia, BAL culture has been shown to reliably reflect the microorganisms of the lungs both qualitatively and quantitatively [21, 22], and BAL is the preferred bronchoscopic sample for aetiological diagnosis of LRTI, according to the European Respiratory Society's recently published guidelines for LRTI [3]. In several studies, BAL culture has been used to establish the aetiology of LRTI [5, 23–29].

TABLE 3 Combined results of tests for *Streptococcus pneumoniae* and *Haemophilus influenzae* in 36 controls

	Results regarding *S. pneumoniae*		Results regarding *H. influenzae*		Controls with the indicated result combination noted[f]
	BAL culture[#]	BAL mPCR[¶]	BAL culture[+]	BAL mPCR[§]	
Controls with lung malignancy[##]	-	+	-	-	1
	+	+	-	+	1
	+	-	+	+	1
	-	-	+	+	3
	-	-	-	+	5
	-	-	-	-	11
Controls with no pathology identified[¶¶]	-	+	-	-	1
	+	-	-	+	1
	-	+	-	+	1
	-		-	+	2
	-	-	-	-	9

BAL: bronchoalveolar lavage; mPCR: multiplex PCR; +: positive; -: negative. [#]: positive, n=3; [¶]: positive, n=4; [+]: positive, n=4; [§]: positive, n=14; [f]: number of patients with the certain combination of results of the previous four columns; [##]: n=22; [¶¶]: n=14.

BAL samples collected by FOB can potentially be contaminated with the oropharyngeal flora by the bronchoscope itself and can cause false positive microbiological results [30, 31]. However, in populations with low rates of carriage in the oropharynx of pathogenic bacteria, some contamination from the oropharyngeal flora may be unimportant. In two previous studies of BAL culture in healthy adults with 15 and 14 subjects, respectively [27, 32], no *S. pneumoniae* or *H. influenzae* could be identified at the detection level of 10^4 cfu·mL^{-1}. KIRKPATRICK *et al.* [31] found *H. influenzae* at a concentration of 10^1 cfu·mL^{-1} in one out of eight healthy subjects, and *S. pneumoniae* in none. This indicates the high specificity of BAL culture for identification of *S. pneumoniae* and *H. influenzae* in LRTI in populations with a low carriage rate of these bacteria. In lower respiratory tract specimens, *S. pneumoniae* and *H. influenzae* are more sensitive to antibiotics than many other bacteria [4]. Thus, in patients treated with antibiotics, it seems reasonable to use a detection level for these two bacteria of $<10^4$ cfu·mL^{-1}. Among the 53 patients not pre-treated with antibiotics in the present study, *S. pneumoniae* or *H. influenzae* was identified at a concentration of $\geqslant 10^4$ cfu·mL^{-1} in 14 (26%) cases and 10^2–10^3 cfu·mL^{-1} in three (5.7%) cases. Due to the low frequency in the latter, a detection level of 10^2 cfu·mL^{-1} was used for BAL culture as well as BAL mPCR in all cases in the study.

In LRTI patients with ongoing antibiotic treatment, BAL mPCR was significantly more often positive for *S. pneumoniae* than BAL culture. The present authors have previously demonstrated that the present urinary antigen test is more often positive in those pre-treated than in those not pre-treated with antibiotics [14]. Thus, mPCR and the urinary antigen test appear useful in patients treated with antibiotics, in order to identify *S. pneumoniae* in patients who will probably give false negative results in their cultures. As FOB is predominantly performed because of treatment failure, BAL mPCR can be a useful routine complement to BAL culture. In the present study, FOB was performed on an unselected LRTI population. However, 66% of the patients had taken antibiotics prior to FOB, and many of them had experienced failure to respond to the outpatient antibiotic treatment, although treatment failure was not defined by the study protocol.

The high rate of antibiotic treatment prior to collection of samples and the low frequency of *S. pneumoniae* aetiology established (14%) in the present study are probably the major reasons for the suboptimal specificity of mPCR for *S. pneumoniae*. Difficulty to estimate reliable specificity is a general problem for new diagnostic tests that are more sensitive than the reference methods. If an sPCR for *S. pneumoniae* had been included in the reference standard, the specificity of mPCR for *S. pneumoniae* would probably have been higher and more correct. However, the usefulness of mPCR for *S. pneumoniae* was supported by the low number of positives in the control group.

When predictive values are calculated for a test, the incidence of the investigated disease is crucial for the level of these values. In a review article of lung aspiration in CAP, SCOTT and HALL [1] found that 48% of adult CAP patients were positive for *S. pneumoniae* in blood culture and/or lung aspirate culture. If the mPCR, with its current sensitivity and specificity for LRTI, was used in a LRTI population with a true frequency of *S. pneumoniae* aetiology of 48%, the positive predictive value would be 81% and the negative predictive value would be 87%.

Although the sensitivity was rather high, the specificity of BAL mPCR for *H. influenzae* was low in the present study. PCR has previously been shown to identify a high rate of colonisation of *H. influenzae* in patients with chronic obstructive pulmonary disease [33], but *H. influenzae* was also identified in 22% by BAL culture and 37% by BAL mPCR in 27 patients who had never smoked. As mPCR has shown a high analytical specificity for *H. influenzae* [12], and as sputum mPCR was positive for *H. influenzae* in only two (7.7%) out of 26 CAP patients with definite aetiologies other than *H. influenzae* in the present authors' previous study [13], the high rate of *H. influenzae* in the present study probably represents colonisation. Hence, it would be interesting to test mPCR on BAL samples from another LRTI population and another control population with a lower smoking frequency.

Owing to a low frequency of *C. pneumoniae* infection during the study period, the sensitivity of BAL mPCR for *C. pneumoniae* could not be evaluated. In the present authors' previous study of 235 patients with CAP [13], three patients were microimmunofluorescence-test positive for *C. pneumoniae* in paired sera; these three patients were also mPCR positive for *C. pneumoniae* in nasopharyngeal secretions. The fact that no control and only one LRTI patient was BAL mPCR positive in the present study indicates that BAL mPCR is probably specific for *C. pneumoniae*. BAL mPCR should be tested on another LRTI population with a higher incidence of *C. pneumoniae* infection to evaluate the usefulness of this assay. BAL mPCR demonstrated an optimal performance for *M. pneumoniae* in the present study (table 1).

Atypical respiratory pathogens have been identified by several previous mPCR protocols [34–37]. However, the present PCR protocol is unique, as it is constructed for identification of two typical and two atypical pathogens, probably the four most common aetiologies of CAP [38].

Due to the high documented aetiological frequencies of the four studied pathogens and due to the high negative predictive values of BAL mPCR (table 1), negative mPCR results may be used to rule out these pathogens as aetiological agents. For a BAL sample that is mPCR positive for a single pathogen, the negative results of the three other pathogens increase the likelihood that the positive test result is truly positive.

As rather low frequencies of *S. pneumoniae* and no *M. pneumoniae* or *C. pneumoniae* were identified in the control group, BAL mPCR results positive for any of these three pathogens can support their role as aetiological agents in LRTI. Consequently, mPCR can be used to support therapeutic decisions in patients with LRTI.

In conclusion, bronchoalveolar lavage multiplex PCR appears to be a useful aetiological tool in lower respiratory tract infection patients, particularly in patients treated with antibiotics. This method could be a valuable supplement to bronchoalveolar lavage culture.

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REFERENCES

1 Scott JA, Hall AJ. The value and complications of percutaneous transthoracic lung aspiration for the etiologic diagnosis of community-acquired pneumonia. *Chest* 1999; 116: 1716–1732.
2 Hedlund J, Strålin K, Örtqvist Å, , Holmberg H, the Community-Acquired Pneumonia Working Group of the Swedish Society of Infectious Diseases, Swedish guidelines for the management of community-acquired pneumonia in immunocompetent adults. *Scand J Infect Dis* 2005; 37: 791–805.
3 Woodhead M, Blasi F, Ewig S, *et al.* Guidelines for the management of adult lower respiratory tract infections. *Eur Respir J* 2005; 26: 1138–1180.
4 Prats E, Dorca J, Pujol M, *et al.* Effects of antibiotics on protected specimen brush sampling in ventilator-associated pneumonia. *Eur Respir J* 2002; 19: 944–951.
5 Hohenthal U, Sipila J, Vainionpaa R, *et al.* Diagnostic value of bronchoalveolar lavage in community-acquired pneumonia in a routine setting: a study on patients treated in a Finnish university hospital. *Scand J Infect Dis* 2004; 36: 198–203.
6 Chastre J, Combes A, Luyt CE. The invasive (quantitative) diagnosis of ventilator-associated pneumonia. *Respir Care* 2005; 50: 797–812.
7 Wheeler J, Freeman R, Steward M, *et al.* Detection of pneumolysin in sputum. *J Med Microbiol* 1999; 48: 863–866.
8 Murdoch DR. Molecular genetic methods in the diagnosis of lower respiratory tract infections. *APMIS* 2004; 112: 713–727.
9 Dorigo-Zetsma JW, Verkooyen RP, van Helden HP, van der Nat H, van den Bosch JM. Molecular detection of *Mycoplasma pneumoniae* in adults with community-acquired pneumonia requiring hospitalization. *J Clin Microbiol* 2001; 39: 1184–1186.
10 Raty R, Ronkko E, Kleemola M. Sample type is crucial to the diagnosis of *Mycoplasma pneumoniae* pneumonia by PCR. *J Med Microbiol* 2005; 54: 287–291.
11 Kuoppa Y, Boman J, Scott L, Kumlin U, Eriksson I, Allard A. Quantitative detection of respiratory *Chlamydia pneumoniae* infection by real-time PCR. *J Clin Microbiol* 2002; 40: 2273–2274.
12 Strålin K, Bäckman A, Holmberg H, Fredlund H, Olcén P. Design of a multiplex PCR for *Streptococcus pneumoniae, Haemophilus influenzae, Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* to be used on sputum samples. *APMIS* 2005; 113: 99–111.
13 Strålin K, Törnqvist E, Kaltoft MS, Olcén P, Holmberg H. Etiologic diagnosis of adult bacterial pneumonia by culture and PCR applied to respiratory tract samples. *J Clin Microbiol* 2006; 44: 643–645.
14 Korsgaard J, Møller JK, Kilian M. Antibiotic treatment and the diagnosis of *Streptococcus pneumoniae* in lower respiratory tract infections in adults. *Int J Infect Dis* 2005; 9: 274–279.
15 Rasmussen TR, Korsgaard J, Møller JK, Sommer T, Kilian M. Quantitative culture of bronchoalveolar lavage fluid in community-acquired lower respiratory tract infections. *Respir Med* 2001; 95: 885–890.
16 Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, eds. Manual of Clinical Microbiology. 7th Edn. Washington, D.C., ASM Press, 1999.
17 Storgaard M, Østergaard L, Jensen JS, *et al. Chlamydia pneumoniae* in children with otitis media. *Clin Infect Dis* 1997; 25: 1090–1093.
18 Tarp B, Jensen JS, Østergaard L, Andersen PL. Search for agents causing atypical pneumonia in HIV-positive patients by inhibitor-controlled PCR assays. *Eur Respir J* 1999; 13: 175–179.
19 Farholt S. *Chlamydia pneumoniae*. PhD thesis. University of Copenhagen, Copenhagen, Denmark, 1996.
20 Nielsen SV, Henrichsen J. Detection of pneumococcal polysaccharide antigens in the urine of patients with bacteraemic and non-bacteraemic pneumococcal pneumonia. *Zentralbl Bakteriol* 1994; 281: 451–456.
21 Chastre J, Fagon JY, Bornet-Lecso M, *et al.* Evaluation of bronchoscopic techniques for the diagnosis of nosocomial pneumonia. *Am J Respir Crit Care Med* 1995; 152: 231–240.
22 Johanson WG Jr, Seidenfeld JJ, Gomez P, de los Santos R, Coalson JJ. Bacteriologic diagnosis of nosocomial pneumonia following prolonged mechanical ventilation. *Am Rev Respir Dis* 1988; 137: 259–264.
23 Sörensen J, Forsberg P, Håkanson E, *et al.* A new diagnostic approach to the patient with severe pneumonia. *Scand J Infect Dis* 1989; 21: 33–41.
24 Thorpe JE, Baughman RP, Frame PT, Wesseler TA, Staneck JL. Bronchoalveolar lavage for diagnosing acute bacterial pneumonia. *J Infect Dis* 1987; 155: 855–861.
25 Feinsilver SH, Fein AM, Niederman MS, Schultz DE, Faegenburg DH. Utility of fiberoptic bronchoscopy in nonresolving pneumonia. *Chest* 1990; 98: 1322–1326.
26 Jimenez P, Saldias F, Meneses M, Silva ME, Wilson MG, Otth L. Diagnostic fiberoptic bronchoscopy in patients with community-acquired pneumonia. Comparison between bronchoalveolar lavage and telescoping plugged catheter cultures. *Chest* 1993; 103: 1023–1027.
27 Dalhoff K, Braun J, Hollandt H, Lipp R, Wiessmann KJ, Marre R. Diagnostic value of bronchoalveolar lavage in patients with opportunistic and nonopportunistic bacterial pneumonia. *Infection* 1993; 21: 291–296.
28 Korsgaard J, Rasmussen TR, Sommer T, Møller JK, Jensen JS, Kilian M. Intensified microbiological investigations in adult patients admitted to hospital with lower respiratory tract infections. *Respir Med* 2002; 96: 344–351.
29 van der Eerden MM, Vlaspolder F, de Graaff CS, Groot T, Jansen HM, Boersma WG. Value of intensive diagnostic microbiological investigation in low- and high-risk patients with community-acquired pneumonia. *Eur J Clin Microbiol Infect Dis* 2005; 24: 241–249.
30 Halperin SA, Suratt PM, Gwaltney JM Jr, Groschel DH, Hendley JO, Eggleston PA. Bacterial cultures of the lower respiratory tract in normal volunteers with and without experimental rhinovirus infection using a plugged double catheter system. *Am Rev Respir Dis* 1982; 125: 678–680.
31 Kirkpatrick MB, Bass JB Jr. Quantitative bacterial cultures of bronchoalveolar lavage fluids and protected brush

catheter specimens from normal subjects. *Am Rev Respir Dis* 1989; 139: 546–548.

32 Cabello H, Torres A, Celis R, *et al.* Bacterial colonization of distal airways in healthy subjects and chronic lung disease: a bronchoscopic study. *Eur Respir J* 1997; 10: 1137–1144.

33 Murphy TF, Brauer AL, Schiffmacher AT, Sethi S. Persistent colonization by *Haemophilus influenzae* in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2004; 170: 266–272.

34 Welti M, Jaton K, Altwegg M, Sahli R, Wenger A, Bille J. Development of a multiplex real-time quantitative PCR assay to detect *Chlamydia pneumoniae, Legionella pneumophila* and *Mycoplasma pneumoniae* in respiratory tract secretions. *Diagn Microbiol Infect Dis* 2003; 45: 85–95.

35 Pinar A, Bozdemir N, Kocagoz T, Alacam R. Rapid detection of bacterial atypical pneumonia agents by multiplex PCR. *Cent Eur J Public Health* 2004; 12: 3–5.

36 Tong CY, Donnelly C, Harvey G, Sillis M. Multiplex polymerase chain reaction for the simultaneous detection of *Mycoplasma pneumoniae, Chlamydia pneumoniae*, and *Chlamydia psittaci* in respiratory samples. *J Clin Pathol* 1999; 52: 257–263.

37 Corsaro D, Valassina M, Venditti D, Venard V, Le Faou A, Valensin PE. Multiplex PCR for rapid and differential diagnosis of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in respiratory infections. *Diagn Microbiol Infect Dis* 1999; 35: 105–108.

38 Marrie TJ. Etiology of community-acquired pneumonia. *In*: Marrie TJ, ed. Community-Acquired Pneumonia. New York, Kluwer Academic/Plenum Publishers, 2001; pp. 131–141.