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

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Short title: Multiplex PCR for bacteria on BAL fluid

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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).

Fibre optic bronchoscopy was performed on 156 hospitalized 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%, yielding sensitivities of 86% for *S. pneumoniae*, 88% for *H. influenzae*, 100% for *M. pneumoniae*, and 0% for *C. pneumoniae*, and specificities of 81% for *S. pneumoniae*, 64% for *H. influenzae*, 100% for *M. pneumoniae*, and 99% for *C. pneumoniae*. In 103 patients with antibiotics taken 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 LRTI patients, BAL mPCR can be useful for identification of *S. pneumoniae*, *M. pneumoniae*, and *C. pneumoniae*. The method appears particularly useful in patients treated with antibiotics.

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

INTRODUCTION

In cases of severe community-acquired lower respiratory tract infection (LRTI) it is desirable to identify the pathogen causing the infection, to choose appropriate antibiotic treatment. Since blood cultures have low sensitivity for LRTI aetiology [1], cultures from the lower respiratory tract are often used. As sputum cultures may be contaminated by the oropharyngeal flora, fibre optic bronchoscopic techniques have been developed to enable collection of lower respiratory tract samples with minimal risk of contamination. At present, a major reason for fibre optic 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, they 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 our knowledge, this has not been evaluated.

One reason for non-response to β-lactam antibiotics in LRTI is atypical pathogens as a cause of the infection. While PCR for *Legionella* species applied to bronchoalveolar lavage (BAL) fluid has been found useful [8], PCR for *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* has not been evaluated on BAL samples. However, since 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 since sputum samples can often not be collected in patients with LRTI, BAL PCR could probably be useful for identification of these two pathogens in LRTI patients.

We 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 analytic sensitivity of 100% (89/89) and a specificity of 99% (167/168) 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 with patients investigated on suspicion of malignancy was included.

MATERIALS AND METHODS

Study subjects

The study was performed prospectively at the Silkeborg County Hospital, Silkeborg, Denmark, the Department of Internal Medicine with 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 described previously [14], all immunocompetent adult patients hospitalised for LRTI

during weekdays between September 1997 and August 2000 were consecutively included in the 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 ≥ 37.6˚C within 48 hours of inclusion in the study) and/or an increased leukocyte count (≥ 11 x 10^9 /L) 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 below 85% with a maximum of 1 litre nasal oxygen were not included. Chest X-ray and spirometry were performed. Demographic data, comorbidity data, 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.

As controls we enrolled adult patients who consecutively underwent FOB for suspected malignancy. Information about prior antibiotics and smoking data were collected.

Bronchoscopy

All patients and controls underwent a standardised FOB with BAL within 24 hours 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 prior chest X-ray was chosen for lavage. In the LRTI patients without X-ray infiltrates and

the control patients, the middle lobe was chosen for lavage. One to three portions of 60 mL of isotonic NaCl 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 Department of Clinical Microbiology, Aarhus University Hospital, Aalborg, Denmark within a maximum of 6 hours from 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 sample. After culture, the BAL fluid was frozen at -20˚C.

BAL samples and throat swabs, which were placed in 2 SP chlamydial transport medium, from the LRTI patients where sent to the Department 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* species [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 at the Department of Clinical Microbiology, Aarhus University Hospital. Non-frozen urine samples were sent to the Department 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 Department of Clinical Microbiology, Örebro University Hospital, Örebro, Sweden for mPCR. A detailed description of the development and procedures of the mPCR is present in a previous paper [12]. mPCR was performed under blinded conditions for simultaneous identification of specific genes for *S. pneumoniae* (*lytA*, 229 base pairs (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′-GTTTCAATCGTCAAGCCGTT-3′; *P1*, 5′-ACTCGGAGGACAATGGTCAG-3′ and 5′-CAAACCCGGTCTTTTCGTTA-3′; *ompA*, 5′-ACACGATGCAGAGTGGTTCA-3′ and 5′-TGTTTACAGAGAATTGCGATACG-3′; and 16S rRNA, 5′-TCCTAAGAAGAGCTCAGAGAT-3′ and 5′-TGATCCAACCGCAGGTTCC-3′. The concentration of all primers was 50 μM. In short, DNA from 0.2-0.5 mL BAL fluid was extracted by the automatic MagNa Pure LC DNA-Isolation system (Roche Diagnostics). Extracted DNA (10 μL) was added to a mixture of 40 μL to give an mPCR mixture volume of 50 μL, including 90 nM of each of the two *lytA* primers and 250 nM of each of the other six primers, 800 μM deoxyribonucleoside triphosphates mix (Perkin-Elmer Applied Biosystem, NJ, USA) (PE), 1.5 U of AmpliTaq Gold DNA Polymerase (PE), and 10x PCR buffer (PE). PCR amplification was performed on a GenAmp PCR system 9600 (PE) with the following parameters: 94°C for 10 min followed by 40 cycles of 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, a 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´-TTGGCGGWTACTCTGTTGCT-3´ and 5´-TGCAGGTTTTTCTTCACCGT-3´). All mPCR negative samples were spiked with a reference strain of *S. pneumoniae* and were rerun 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 sample for *S. pneumoniae* and *H. influenzae*, and 10^3 genome copies or inclusion-forming units/mL sample for *M. pneumoniae* and *C. pneumoniae* [12].

Criteria for aetiologies and exclusion of aetiologies

Our criteria for LRTI aetiologies were: *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 approved by the local ethical committee and all participating patients gave written consent.

RESULTS

Patients and controls

During the study period 167 patients fulfilled the study criteria for LRTI. As 8 of them declined participation, 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 years (range 26-90 years), 48 patients (31%) were current and 62 (40%) were previous smokers, and the mean pack-years history in the current and previous smokers was 27 years. A chronic lung disease was documented in 72 patients (46%), and the mean forced expiratory volume in 1 s on day 7 from inclusion was 58% of predicted. New X-ray infiltrates were identified in 87 LRTI patients (56%). The median interval between illness onset and FOB was 8 days, FOB was performed within two weeks of illness onset in 72% of the patients, and FOB was performed within 24 hours of hospitalisation in all patients. Any antibiotics had been taken within 7 days prior to FOB in 103 patients (66%). The median length of hospital stay was 8 days (range 1-40 days). One patient died within 30 days from admission.

Of 51 controls included, 12 were excluded as they were diagnosed as having pulmonary infection with X-ray 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 2 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 years (range 30-77 years). Thirty-two of them (89%) were current or previous smokers.

Identification of pathogens in the patients with lower respiratory tract infection

In 55 LRTI patients (35%) any of the four studied pathogens was considered to be the aetiology, i.e., *S. pneumoniae* in 18 patients (12%), *H. influenzae* in 30 patients (19%), both *S. pneumoniae* and *H. influenzae* in 2 patients (1.3%), *M. pneumoniae* in 5 patients (3.2%), and *C. pneumoniae* in no patient.

S. pneumoniae was identified by blood culture in 6 patients (3.9%) of 152 tested, by urinary antigen test in 9 (6.3%) of 142 tested, by BAL culture in 10 (6.4%) of 156 tested, and by sputum culture in 3 (5.3%) of 56 tested. Two of those with positive sputum culture were also BAL culture positive for *S. pneumoniae*. *H. influenzae* was identified by BAL culture in 31 patients (20%) of 156 tested and by sputum culture in 6 (11%) of 56 tested, but in no blood culture. Five of the 6 patients with positive sputum culture were also BAL culture positive for *H. influenzae*. *M. pneumoniae* was identified in 5 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* species by urinary antigen test (154 tested), BAL sPCR (155 tested), or throat swab sPCR (156 tested). No patient was blood culture positive 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* species in 5 patients, *Neisseria meningitidis* in 3 patients, and *Enterococcus faecalis* in 1 patient. sPCR identified *C. psittaci* in one patient.

Streptococcus pneumoniae

S. pneumoniae was an aetiological agent in 22 patients (14%) by the conventional tests, and was identified by BAL mPCR in 44 (28%) of the 156 LRTI patients. With all test included, *S. pneumoniae* was identified in 30% of the LRTI patients. Table 2 demonstrates moderate to high sensitivity and negative predictive value, but low specificity and positive predictive value of BAL mPCR for *S. pneumoniae*. Among the 6 patients with pneumococcal bacteraemia, *S. pneumoniae* was identified by BAL mPCR in all 6 patients and by BAL culture in one patient. Likewise, of the 9 patients with positive urinary antigen test, *S. pneumoniae* was identified in 7 by BAL mPCR and in none by BAL culture. Among the 87 patients with X-ray infiltrates, mPCR for *S. pneumoniae* had a sensitivity of 94% (16/17) and a specificity of 73% (51/70).

Haemophilus influenzae

H. influenzae was an aetiological agent in 32 patients (21%) 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 2). Among the 87 patients with X-ray infiltrates, mPCR for *H. influenzae* had a sensitivity of 84% (16/19) and a specificity of 62% (42/68).

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 5 LRTI patients (3.2%). There was total agreement between BAL sPCR, throat swab sPCR, and BAL mPCR for identification of *M. pneumoniae* in the present study (Table 2). 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 relation between the different tests for *S. pneumoniae* and *H. influenzae* is shown in table 1. Both of these pathogens were identified in 18 patients; *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* species 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 (non-significant). A bacterial concentration of $\geq 10^4$ CFU/mL in BAL culture was identified in 6 of 7 patients with *S. pneumoniae* identified and in 8 of 10 patients with *H. influenzae* identified.

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-square test), and *H. influenzae* in 20% (n=21) and 50% (n=51), respectively (non-significant), of the patients. A bacterial concentration of $\geq 10^4$ CFU/mL in BAL culture was identified in 1 of 3 patients with *S. pneumoniae* identified and in 16 of 21 patients with *H. influenzae* identified. One LRTI patient with pneumococcal bacteremia 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 5 patients with *M. pneumoniae* aetiology, 3 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 6 controls (17%) and *H. influenzae* in 14 controls (39%). In the controls with lung malignancy, *S. aureus* was identified in 3 controls and Gram-negative enteric bacilli in 2 controls. *S. aureus* was also identified in one control without pathology identified. No control was mPCR positive for *M. pneumoniae* or *C. pneumoniae*.

PCR inhibition

No PCR inhibition was identified in any BAL mPCR analysis.

DISCUSSION

In pneumonia, BAL culture has been shown to reliably reflect the micro-organisms 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].

BAL samples collected by FOB can potentially be contaminated with the oropharyngeal flora by the bronchoscope itself and 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. Kirkpatrick et al. [31] found *H. influenzae* at a concentration of 10^1 CFU/mL in one of 8 healthy subjects, and *S. pneumoniae* in none. This indicates 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 antibiotic treated patients, it seems reasonable to use a detection level for these two bacteria lower than

10^4 CFU/mL, which is often used. Among the 53 patient not pre-treated with antibiotics in the present study, *S. pneumoniae* or *H. influenzae* was identified at a concentration of $\geq 10^4$ CFU/mL in 14 cases (26%) and 10^2-10^3 CFU/mL in 3 cases (5.7%). Due to the low latter frequency, a detection level of 10^2 CFU/mL was used for BAL culture as well as BAL mPCR in all cases of the study.

In the LRTI patients with ongoing antibiotic treatment, BAL mPCR was significantly more often positive for *S. pneumoniae* than BAL culture. We 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 urinary antigen test appear useful in antibiotic treated patients, in order to identify *S. pneumoniae* in patients who are probably false negative in the cultures. Since 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 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 community-acquired pneumonia (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 it's current sensitivity and specificity for LRTI would be 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 analytic specificity for *H. influenzae* [12], and as sputum mPCR was positive for *H. influenzae* in only 2 of 26 CAP patients (7.7%) with definite aetiologies other than *H. influenzae* in our 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 lower frequency of smoking.

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 our previous study of 235 patients with CAP [13], three patients were microimmunofluorescence test positive for *C. pneumoniae* in paired sera and they were all mPCR positive for *C. pneumoniae* in nasopharyngeal secretions. The fact that only one LRTI patient and no control was BAL

mPCR positive in the present study indicates that BAL mPCR for *C. pneumoniae* is probably specific. BAL mPCR should be tested on another LRTI population with higher incidence of *C. pneumoniae* infection to evaluate the usefulness of the assay.

BAL mPCR demonstrated an optimal performance for *M. pneumoniae* in the present study (Table 2).

Atypical respiratory pathogens have been identified by several previous multiplex PCR 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 2), 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, BAL mPCR appears to be a useful aetiological tool in LRTI patients, particularly in antibiotic treated patients. The method could be a valuable supplement to BAL 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, and 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, Huchon G, Leven M, Örtqvist Å, Schaberg T, Torres A, van der Heijden G, Verheij TJ. Guidelines for the management of adult lower respiratory tract infections. *Eur Respir J* 2005: 26: 1138-1180.
4. Prats E, Dorca J, Pujol M, Garcia L, Barreiro B, Verdaguer R, Gudiol F, Manresa F. 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, Meurman O, Rantakokko-Jalava K, Nikoskelainen J, Kotilainen P. 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, Henderson K, Lee MJ, Piggott NH, Eltringham GJ, Galloway A. 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. 7. ed. ASM Press, Washington, D.C., 1999.
17. Storgaard M, Østergaard L, Jensen JS, Farholt S, Larsen K, Ovesen T, Nodgaard H, Andersen PL. *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, Calvat S, Dombret MC, al Khani R, Basset F, Gibert C. 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, Maller R, Sederholm C, Sörén L, Carlsson C. 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, El-Ebiary M, Puig de la Bellacasa J, Xaubet A, Gonzalez J, Agusti C, Soler N. 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. Kluwer Academic / Plenum Publishers, New York, 2001; pp. 131-141.

TABLE 1 The 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 result combination No.
Blood culture and/or urinary antigen test (positive, n=12)	BAL culture and/or sputum culture (positive, n=11)	BAL mPCR (positive, n=44)	BAL culture and/or sputum culture (positive, n=32)	BAL mPCR (positive, n=73)	
+	-	+	-	-	6
-	+	+	-	-	8
-	+	-	-	-	1
-	-	+	-	-	14
+	+	+	-	+	1
+	-	+	+	+	1
+	-	+	-	+	2
+	-	-	+	+	1
+	-	-	-	+	1
-	+	+	-	+	1
-	-	+	+	+	3
-	-	+	+	-	2
-	-	+	-	+	6
-	-	-	+	+	23
-	-	-	+	-	2
-	-	-	-	+	34
-	-	-	-	-	49
No test was performed	-	-	-	-	1

BAL: Bronchoalveolar lavage; mPCR: multiplex PCR.

TABLE 2 Performance of multiplex PCR (mPCR) applied to bronchoalveolar lavage (BAL) in 156 adults with lower respiratory tract infection

Species	Reference tests	Sensitivity [#]	Specificity [¶]	Positive predictive value [+]	Negative predictive value [§]
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)

[#] 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 3 The combined results of tests for *Streptococcus pneumoniae* and *Haemophilus influenzae* in 36 controls

	Results regarding *S. pneumoniae*		Results regarding *H. influenzae*		Controls with the result combination No.
	BAL culture (positive, n=3)	BAL mPCR (positive, n=4)	BAL culture (positive, n=4)	BAL mPCR (positive, n=14)	
Controls with lung malignancy (n=22)	-	+	-	-	1
	+	+	-	+	1
	+	-	+	+	1
	-	-	+	+	3
	-	-	-	+	5
	-	-	-	-	11
Controls with no pathology identified (n=14)	-	+	-	-	1
	+	-	-	+	1
	-	+	-	+	1
	-	-	-	+	2
	-	-	-	-	9

BAL: Bronchoalveolar lavage; mPCR: multiplex PCR.