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Molecular diagnostics for bacterial infections in bronchoalveolar lavage – a case-control, pilot study

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Summary

QUESTIONS UNDER STUDY: The differentiation between infectious and noninfectious pulmonary complications is challenging. Rapid, accurate microbiological results may allow appropriate antibiotic therapy, withholding or adapting antibiotics, and thus reducing costs and risks of empirical antibiotic therapy. The objective of this proof-of-concept pilot study was to investigate the diagnostic yield of a new polymerase chain-reaction (PCR) and microarray-based rapid molecular diagnostic assay and compare the results to conventional microbiology cultures and clinical judgment.

METHODS: Bronchoalveolar lavage specimens were obtained from 35 patients undergoing bronchoscopy for diagnostic reasons. Cases (n = 22) consisted of patients with suspicion of pulmonary bacterial infection. Controls (n = 13) were identified among patients undergoing bronchoscopy for indications other than suspicion of infection.

RESULTS: Demographics were similar in cases and controls. The majority (73%) of patients with pulmonary infection were on empirical antibiotic therapy. Among the 22 cases, bacteria were identified by means of PCR in 77% (n = 17) as compared with 41% (n = 9) by culture (p = 0.030). In contrast, controls yielded a PCR positive result in 45% (n = 7), as compared with no positive cultures (p = 0.005). Compared with culture results, PCR had a sensitivity of 87.5% (95% confidence interval [CI] 47.4–97.9) and specificity of 28.6% (95% CI 8.6–58.1) to diagnose bacterial infection. Compared with clinical judgment, corresponding figures were 77.3% (95% CI 54.5–91.1) and 46.2% (95% CI 19.3–74.8), respectively.

CONCLUSION: The PCR- and microarray-based rapid molecular diagnostic assay offers an alternative to conventional cultures for detection of potentially pathogenic bacteria in bronchoalveolar lavage of patients with pneumonia. However, the clinical relevance is unclear as it may also detect colonisers in patients without a corresponding infection.

Key words: *molecular diagnostics; bacterial infections; bronchoalveolar lavage; PCR; clinical judgment*

Abbreviations

PCR	polymerase chain reaction
BAL	bronchoalveolar lavage
EKBB	Ethikkommission beider Basel (ethic commission Basel)
CT	computer tomography
Spp	species
NAT	nucleic acid amplification testing
DNA	deoxyribonucleic acid
MRSA	multiresistant *Staphylococcus aureus*
SPSS	Statistical Package for Social Science
LRTID	lower respiratory tract infectious disease
URTID	upper respiratory tract infectious disease
COPD	chronic obstructive pulmonary disease
HSCT	human stem cell transplantation
HIV	human immunodeficiency virus
ILD	interstitial lung disease
IPF	idiopathic pulmonary fibrosis
NLR	negative likelihood ratio
PLR	positive likelihood ratio

Introduction

Pulmonary infections are the most common cause of morbidity and mortality worldwide [1]. Their accurate and rapid diagnosis is pivotal as it influences infection control measures, specific anti-microbial therapy, and outcome [2–5]. Noninfectious pulmonary complications tend to be less common than infectious complications in hospitalised patients. In fact, fewer than half of all potentially treatable, nonbacterial pulmonary infections have been diagnosed prior to death [6, 7].

The differentiation between infectious and noninfectious complications is challenging, particularly in immune-compromised patients [8]. Clinical parameters and laboratory values are sensitive, but nonspecific for bacterial conditions [9]. Radiographic signs commonly associated with pneumonia (e.g. infiltrates) do not necessarily provide evidence of bacterial infectious processes. Microbiological

examination of bronchoalveolar lavage (BAL) has been established as a reliable technique for the targeted diagnosis of pulmonary infections. However, results of BAL cultures are not immediately available. Bacterial cultures commonly need 2 to 5 days to provide a definitive result. Moreover, because of the perceived severity of the baseline condition and worsened prognosis associated with delay in antibiotic therapy, direct respiratory tract sampling usually occurs after antibiotic therapy has been instituted [10, 11]. Ongoing empirical antibiotic therapy limits the microbiological yield of BAL, which ranges widely from 15 to 93% [5, 12–14] and is cost-intensive [15].

Rapid, accurate microbiological results might allow appropriate antibiotic therapy to be instituted withholding or adaptation of antibiotics, and thus a decrease in costs and risks of empirical antibiotic therapy [1, 16, 17]. In a previous study, the polymerase chain reaction (PCR) and microarray-based Prove-it™ Sepsis assay was used for detection of bloodstream infections in septic patients with a reported sensitivity of 95% and specificity of 99% [3]. The current study aimed to evaluate the diagnostic yield of this new, rapid, broad-range assay for bacterial infections in BAL. The case cohort consisted of patients with suspected pulmonary infection undergoing diagnostic bronchoscopy and was compared to a control group of patients undergoing diagnostic bronchoscopy without suspicion of pulmonary infection.

Methods

Study

This was a single-centre, case-control, observational pilot study performed at the University Hospital Basel, a 784-bed tertiary care institution located in Basel, Switzerland. The study was approved by our institutional review board (EKBB 120/10) and subjects provided written informed consent.

Patients

Patients undergoing diagnostic bronchoscopy between February 2011 and May 2011 were considered for eligibility. Inclusion criteria included: (a) age >18 years; (b) suspicion of pulmonary infection; (c) absence of other pulmonary pathologies such as cystic fibrosis. Patients of the control group were selected during the same time period. Inclusion criteria were: (a) age >18 years; (b) no suspicion of pulmonary infection; (c) absence of other pulmonary pathologies such as cystic fibrosis. Exclusion criteria for both patients and controls were: (a) requirement for therapeutic bronchoscopy; (b) failure to provide informed consent.

Suspicion of pulmonary infection was defined by the clinical presentation (cough, sputum, dyspnoea / shortness of breath, wheezing and/or fever), and/or a new or progressive radiological finding as judged by the attending physician. All patients underwent a thorough physical examination and laboratory sampling. Radiographic examination (chest x-ray and/or chest computed tomography [CT]-scan) was performed before flexible bronchoscopy. Immuno-compromised conditions were defined by the presence of a malignant comorbidity, such as haematological disease, systemic steroid therapy >10 mg/d and/or ongoing chemotherapy.

The control group consisted of patients undergoing elective bronchoscopies for investigational procedures to exclude malignancy, to differentiate a radiological suspicion of interstitial lung disease (ILD) and for a bronchial wash.

Bronchoscopy with bronchoalveolar lavage

Bronchoscopies were performed trans-nasally or trans-orally with the patients in a semi-recumbent position by a total of five pulmonary fellows under close supervision of four pulmonary attending physicians, as previous described [18, 19]. Pulse oxymetry was recorded continuously during the procedure and automated noninvasive blood pressure measurements were monitored every 5 minutes. Supplemental oxygen was offered at 4 l/min via nasal cannula to all patients. In the case of desaturation, ≤90%, oxygen delivery was increased to 6 l/min [20].

Bronchoalveolar lavage was performed by instillation of sterile NaCl 0.9% (3 x 50 ml) via the working channel of the bronchoscope into the affected lobe or segment, according to the radiological findings. In patients with diffuse processes, the middle lobe or lingula was selected for lavage. The retrieval of fluid was achieved by suction.

The collected sampling fluid was analysed by use of microscopy (differential cell counts) and conventional microbiology (stains and cultures for bacteria, including mycobacteria and fungi, as appropriate). Quantitative bacterial findings were reported with a cut-off limit of $>10^4$ colony forming units, considered to represent bacterial infection. The presence of mouth flora was defined by the presence of at least two of the following bacteria: *Viridans Streptococcus* group (except *Streptococcus pneumoniae* and *Enterococcus* spp.), coagulase-negative *Streptococcus*, *Neisseria* spp., nonhaemolytic *Streptococcus* spp., or coryneform bacteria without evidence of Gram-negative organisms.

Additionally, nucleic acid amplification testing (NAT) (RespiFinder-22, Pathofinder, Maastricht, The Netherlands) including adenovirus; *Bordetella pertussis*; *Chlamydia pneumonia*; coronavirus NL63, -OC43, -229E; influenza A and B; *Legionella* sp., parainfluenza 1, 2, 3, 4; respiratory syncytial virus A and B; and human metapneumovirus; rhino-/enterovirus was performed.

Rapid broad-range PCR and microarray-based nucleic acid amplification testing assay

Rapid broad-range PCR and microarray-based NAT assay for 12 Gram-positive and 19 gram-negative bacteria (table 1) was performed by using the DNA extract from 500 µl of pretreated bronchoalveolar lavage fluid according to the manufacturer's instructions (Prove-it™ Sepsis assay, Mobidiag, Helsinki, Finland). Among pathogens, *Neisseria meningitides* could also be detected and this was of particular importance since it is a rare but well-described pathogen causing acute pneumonia in adults.

Sample preparation

The samples were transferred onto dry ice and frozen to –20 °C. After thawing at room temperature, samples were vortexed for 10 seconds and 500 µl of sample was trans-

ferred to a fresh sterile tube. Then 20 µl of 20 mg/ml proteinase K solution (Roche, FR) was added to 500 µl of BAL sample, and samples were incubated for 20 min at 60 °C under 400 rpm agitation. Proteinase K was inactivated by heating the samples to 95 °C and incubating for 10 min without agitation. Samples were cooled to room temperature before DNA extraction.

DNA extraction

DNA was extracted with NucliSENS® easyMAG® instrument (bioMérieux, FR) according to the manufacturer's instructions. A total of 500 µl of the pretreated BAL samples were added to 2 ml of easyMAG® Lysis Buffer, vortexed and incubated for 10 min at room temperature. Thereafter, the entire sample was transferred to the extraction vessel and the "Generic 2.0.1." program was used with an elution volume of 25 µl.

Prove-it™ Sepsis assay

The extracted DNA was subjected to PCR amplification and microarray hybridisation on the Prove-it™ StripArray platform according to the manufacturer's instructions (Mobidiag, Helsinki, Finland). In short, 1.5 µl DNA extract was used for PCR in a total reaction volume of 15 µl. Negative control reactions (PCR grade water as sample) and positive control reactions (DNA extract of multiresistant *Staphylococcus aureus* [MRSA]) were included in the test series. Immediately after PCR, 3 µl of the PCR product was subjected to a single microarray containing bacterial DNA capture probes of all bacteria included in the test panel (table 1). It should be emphasised that this assay does not constitute a respiratory bacterial panel but rather a broad septic panel. The results were automatically analysed with Prove-it™ Advisor software. The turnaround time for the Prove-it™ Sepsis assay is about 3.5 hours. Together with the sample pretreatment and DNA extraction, the results were available during the same working day.

Statistical analysis

Differences in dichotomous variables were evaluated using the chi-square test or Fischer's exact test, as appropriate. Normally distributed parameters were analysed using the Student's t-test for equality of means. All other continuously, non-normally distributed parameters were evaluated using the non-parametric Mann–Whitney U test or Kruskal-Wallis test, as appropriate. Sensitivity, specificity, and positive and negative likelihood ratios for the molecular diagnostic assay were calculated by using conventional cultures and clinical judgment as the gold standards for the diagnosis of bacterial infection. All tests were two-tailed; a p-value of <0.05 was considered significant. Results were expressed as mean (standard deviation) or median (interquartile range) unless otherwise stated. Data analysis was conducted with the Statistical Package for Social Sciences (SPSS Inc, version 22 for Windows) program.

Results

Study subjects

A total of 35 patients were included in this single centre, case-controlled pilot study. Baseline characteristics of cases (n = 22) and controls (n = 13) are depicted in table 2. The case group was defined by a clinical suspicion of a respiratory tract infection. The control group consisted of a cohort of patients without suspicion of respiratory tract infection. In this control group, we included patients who underwent bronchoscopy following suspicion of pulmonary malignancy or metastatic diseases or had radiological features of interstitial lung diseases without any clinical hint of an infection of upper respiratory tract infectious disease (URTID) / lower respiratory tract infectious disease (LRTID).

Age and biometrics were similar in cases and controls. As expected, patients presented more commonly with fever, higher leucocyte counts and circulating C-reactive protein levels and more radiological abnormalities. In the case group, 72.7% of the patients were subjected to a CT scan. We detected infiltration in 50% of the patients, pulmonary nodules in 13.6%, a typical idiopathic pulmonary fibrosis (IPF) pattern in 9%, bronchiectasis in 9%, cavernous process in 9%, atelectasis in 4.5% and unilateral pneumothorax in 4.5%. The remaining 13.6% of the patients of this group were subjected to X-ray. Infiltration was detected in all these patients.

In the control group, 77% of the patients were subjected to a CT scan. We detected infiltration in 15.4% of the cases, pulmonary nodules in 23.2%, an IPF pattern in 15.4% and atelectasis in 7.7%. The remaining 15.4% of the patients in the control group were subjected to X-ray. Infiltration was detected in 7.7% of these patients and nonremarkable findings in 7.7%.

Cytological analysis of the BAL samples of the case group, revealed that in a total cell count of 486.58 x 10^6/l, 54.3% were macrophages, 17% were neutrophils, 28.05% were lymphocytes and 1.7% were eosinophils. In the control group, the total cell count was 106.13 x 10^6/l, with 89.77%

Table 1: Targeted bacterial species with Prove-itTM Sepsis nucleic acid amplification testing (NAT) assay.

Gram-positive	Gram-negative
Clostridium perfringens	*Acinetobacter baumannii*
Enterococcus faecalis	*Enterobacter aerogenes*
Enterococcus faecium	*Enterobacter cloacae*
Listeria monocytogenes	*Escherichia coli*
Propionibacterium acnes	*Haemophilus influenzae*
Staphylococcus aureus	*Kingella kingae*
Staphylococcus epidermidis	*Klebsiella oxytoca*
Streptococcus agalactiae	*Klebsiella pneumonia*
Streptococcus dysgalactiae subspecies *equisimilis*	*Neisseria meningitidis*
Streptococcus pneumoniae	*Proteus mirabilis*
Streptococcus pygenes	*Proteus vulgaris*
Coagulase-negative *Staphylococcus*	*Pseudomonas aeruginosa*
	Salmonella enteridis subspecies *enteritica*
	Serratia marcescens
	Stenotrophomonas maltophilia
	Bacteroides fragilis group
	Campylobacter jejuni/coli
	Enterobacteriaceae species
	Neisseria species, non-meningitidis

macrophages, 5.45% neutrophils, 8.59% lymphocytes and no eosinophils. Cytological data confirmed a normal cell distribution in adequate BAL samples.

Chronic obstructive pulmonary disease (COPD) and bronchial asthma were the most common comorbidities. Immunosuppression was present in 32% of suspected respiratory infections (cases) and 23% of noninfected cases (controls). The majority (73%) of patients with a suspicion of pulmonary infection were on empirical antibiotic therapy at the time of bronchoscopy. Most macroscopic bronchoscopic findings were suggestive of infection among cases (purulent secretion in 55%, mucosal redness in 41%, and increased mucus secretion in 27%), as compared with controls (mucosal redness in 38.5%).

Diagnostic yield of bacterial nucleic acid amplification testing assay

In cases, bacteria were identified by Prove-it™ Sepsis NAT assay in 77% of the patients (n = 17), compared with 40.9% of the patients (n = 9) identified positive by conventional cultures (p =0.0305). In contrast, a bacterial identification was reported in 45% (n = 7) of controls with the NAT assay compared with no positive cultures (p = 0.005). Table 3 depicts microbiological and NAT results of both assays in cases and controls. In addition to the organisms presented in the table, *Achromobacter xyloxidans* was detected in two cases by culture. *Mycobacterium avium intracellulare* was detected in one case, which also tested positive for *Haemophilus influenzae*. Compared with culture, NAT was superior for the detection of potentially pathogenic bacteria such as *Haemophilus influenzae* (six divergent cases), *Moraxella catarrhalis* (two divergent cases) and *Stenotrophomonas maltophilia* (one case). Conversely, *Staphylococcus epidermidis*, a potential commensal microorganism, was detected solely by the NAT assay in three cases and four controls. There is no separate listing of *Staphylococcus epidermidis* in bacterial cultures since it is classified as mouth flora, as previously described. Among controls, NAT detected *Haemophilus influenzae* in three patients and *Streptococcus pneumoniae* in two patients.

Viruses were detected in 27% (n = 6) among cases and 15% (n = 2) among controls. The most common viruses were rhinovirus (n = 3), cytomegalovirus (n = 2) and influenza virus(n = 1) in cases, and respiratory syncytial virus and rhinovirus (one each) in controls. Fungal microorganisms were present in 12 cases (7 *Candida* spp., 3 fungal moulds, 2 *Aspergillus* spp.) and none of the controls.

The detailed diagnostic performance of rapid broad-range PCR and microarray-based assay using conventional cultures and the clinical judgment as the gold standard is presented in table 4. The sensitivity of NAT / clinical judgment is better than NAT/culture. Regarding the specificity, both methods showed low specificity; however, the specificity of the NAT test is better than that of conventional culture.

Discussion

In this pilot study, we analysed for the first time the diagnostic performance of a new, PCR- and microarray-based rapid molecular diagnostic assay in bronchoalveolar lavage of cases with suspicion of pulmonary infection and of controls, and compared the results to conventional microbiology cultures and clinical judgment. The control group comprised of subjects without any hint for respiratory tract infection who underwent bronchoscopy in order to investigate pulmonary malignancy or metastatic diseases or because of radiological features indicating interstitial lung

Table 2: Baseline characteristics of cases and controls.

	Cases (n = 22)	**Controls (n = 13)**
Age (years)	61.4 ± 15.2	59.9 ± 16.5
Gender (female)	36%	54%
Weight (kg)	73.8 ± 14.6	72.3 ± 16.5
Height (cm)	168.1 ± 8.9	166.3 ± 8.0
Fever	64%	9%
Previous antibiotic therapy	73%	15%
Underlying diseases:		
Immunosuppressed	31.8%	23.1%
Allogenic HSCT	4 (18.2%)	1 (7.6%)
COPD/asthma	12 (54.5%)	6 (46.2%)
Lung transplantation	2 (9%)	0 (0%)
Liver transplantation	1 (4.5%)	0 (0%)
Interstitial lung disease	2 (9%)	2 (15.4%)
HIV	1 (4.5)	0 (0%)
Pulmonary nodule	0 (0%)	4 (30.8%)
Solid tumour	2 (9%)	0 (0%)
Indication for bronchoscopy:		
Suspicion for infectious disease	100%	0%
ILD	0%	15.4%
Cough/asthma/COPD	54.5%	53.8%
Pulmonary nodule work-up	0%	30.8%
Laboratory results:		
Leucocytes ($\times 10^9$/l)	11.39	9.15
Neutrophils ($\times 10^9$/l)	9.6	6.56
C-reactive protein (mg/ l)	101.48	5.24

CT = computed tomography; HSCT = human stem cell transplantation; COPD = chronic obstructive pulmonary disease; HIV = human immunodeficiency virus; HSCT = human stem cell transplantation; ILD = interstitial lung disease; IPF = interstitial pulmonary fibrosis

diseases. These subjects were outpatients since we aimed to investigate community acquired LRTIDs. The number of outpatients who had the above characteristics was very low and, therefore, the number of subjects was lower in the control group than in the group of cases.

We report that a molecular diagnostic assay, such as the NAT assay, offers relative advantages, as compared with conventional cultures, for the detection of potentially pathogenic bacteria in bronchoalveolar lavage of patients with suspicion of LRTID. However, the NAT assay, which is a highly sensitive assay, may also provide a positive diagnosis in a population of patients without evidence of bacterial infection. This apparent discrepancy between the NAT assay and conventional bacteriology may be due to the following reasons: (a) there may be a contamination of the samples with either skin and/or oropharynx pathogens, or pathogens of chronic airway colonisation, as observed in COPD; (b) it could also be postulated that previous antibiotic use may hinder bacterial growth in a conventional bacteriology culture, whereas, in such cases, the NAT assay would provide an apparent advantage; (c) the NAT assay may also detect DNA from nonliving bacteria, which would give a negative result after conventional bacteriological culture [21].

The above reasons may explain the high rate (45%) of positive results obtained with the NAT assay in controls, even though there was no suspicion of bacterial infection. Clinical judgment is not 100% accurate for ruling bacterial infection in or out. Therefore, even in these cases, the presence of a bacterial infection would be possible. Correspondingly, the absence of bacterial growth in the cases does not rule out the presence of bacterial infection, as, particularly in individuals on antibiotics, bacterial growth might be absent despite true infection. Since molecular methods have high sensitivity and are able to detect small amounts of pathogen DNA and taking into account that the respiratory tract is not necessarily sterile even in individuals without true clinical acute infection, as in COPD and bronchiectasis, one could potentially overdiagnose infection of the respiratory system. Thus, the interpretation of PCR results requires accurate consideration of clinical presentation and microbiological data. Additionally, clinically relevant cut-offs for each bacterium (depending on pathogenicity) should be determined and validated. Therefore, based on the clinical presentation, most of the formal pathogens detected by the NAT assay in our controls could be categorized under contamination or commensals.

In this respect, we found that 40% of the control subjects were colonised by *Streptococcus pneumoniae* and *Haemophilus influenzae*, both potentially pathogenic microorganisms that are known to colonise the oropharyngeal tract. *Haemophilus influenzae* was evident in COPD/asthma cases and in one patient with immunosuppression while suffering from an ILD. Although, as a result of the lack of clinical symptoms, bacterial detection in the BAL might not necessarily lead to antibiotic treatment at present, colonisation by *Streptococcus pneumoniae* and *Haemophilus influenzae* is a typical feature of chronic lung diseases such as COPD and is thought to be linked to clinically relevant outcomes [22]. Indeed, all five controls with either *Streptococcus pneumoniae* or *Haemophilus influenzae findings* were found to present COPD and/or bronchiectasis or to be immunosuppressed with steroids. The PCR detection of *Haemophilus influenzae* in the cases group by NAT included two patients with post-stenotic pneumonia while suffering from bronchus carcinoma, two immunosuppressed patients with status after lung transplantation and immunodeficiency syndrome with bronchiectasis and three patients with COPD/asthma. In controls, the NAT detected *Haemophilus influenzae* in one patient with suspicion of malignancy, in one patient with COPD/asthma and in one patient under immunosuppression while suffering from ILD.

The detection of *Haemophilus influnzae* by the NAT assay is very promising since it is a very frequent pathogen in the airways associated with high morbidity. Furthermore, since some atypical *H. influenza* isolates are producing beta-lactamase, the information for its presence or absence

Table 3: Detection of bacterial pathogens in bronchoalveolar lavage samples of cases and controls by the Prove-it™ Sepsis NAT assay (PCR) and culture.

Microorganism	**Cases n = 22**		**Controls n = 13**	
	PCR	**Culture**	**PCR**	**Culture**
Any bacteria (n, %)	17 (77%)	9 (40.9%)	7 (45%)	0
Gram positive (n, %)				
Enterococcus spp.	3 (13.6%)	1 (4.5%)	0	0
Streptococcus pneumoniae	2 (9.1%)	2 (9.1%)	2 (15.4%)	0
Staphylococcus aureus	1 (4.5%)	1 (4.5%)	0	0
Coagulase-negative *Staphylococcus*	5 (22.7%)	2 (9.1%)	4 (30.8%)	0
Gram negative (n, %)				
Haemophilus influenzae	7 (32%)	1 (4.5%)	3 (23.1%)	0
Proteus vulgaris	1 (4.5%)	1 (4.5%)	0	0
Stenotroph maltophilia	1 (4.5%)	0	0	0
Pseudomonas aeruginosa	1 (4.5%)	2 (9.1%)	0	0
Moraxella catarrhalis	2 (9.1%)	0	0	0

PCR = polymerase chain reaction; spp. = species; NAT = nucleic acid amplification testing

Table 4: Diagnostic performance of Prove-it™ Sepsis NAT assay in bronchoalveolar lavage of 22 cases and 13 controls compared with culture and clinical judgment.

Comparison with	**Sensitivity (CI)**	**Specificity (CI)**	**NLR (CI)**	**PLR (CI)**
culture	87.5% (47.4–97.9)	28.6% (8.6–58.1)	0.44 (0.06–3.27)	1.22 (0.8–1.87)
clinical judgment	77.3% (54.5–91.1)	46.2% (19.3–74.8)	0.49 (0.19–1.30)	1.44 (0.8–2.49)

CI = 95% confidence interval; NAT = nucleic acid amplification testing; NLR = negative likelihood ratio; PLR = positive likelihood ratio

may offer the possibility to switch to a narrow spectrum agent such as amoxicillin and to reduce the use of macrolides and quinolones wherever possible.

Colonisation of the respiratory tract should be strictly distinguished from an acute LRTI/ URTI. The indication and duration of antibiotic therapy should be designated according to the pathogens associated with acute illness but not for commensals or colonisers. The early demarcation of these three groups could eventually optimise therapy costs towing to the right choice of the most appropriate antibiotic and could decrease antibiotic resistance by narrowing the antibiotic spectrum.

While the bacterial multiplex NAT could be a promising tool to diagnose bacterial infection in BAL of patients with suspected pulmonary bacterial infection under current treatment with antibiotics, there are some drawbacks that need to be taken into consideration. First, in two cases, the NAT reported false negative results for potentially pathogenic bacteria (*Pseudomonas aeruginosa* and Enterobacteriaceae) compared with microbiological culture. As both patients were on antibiotics, the consequences of overlooking the presence of these microorganisms remained unknown. Unfortunately, *Pseudomonas aeruginosa* can be both a coloniser or an acute pathogen in patients with chronic diseases such as COPD or bronchiectasis. Ideally, the NAT assay should not miss this relevant pathogen. Therefore, the primers have to be sensitised for a low threshold of this pathogen.

In our study, we provide evidence that the sensitivity of NAT / clinical judgment was better than NAT/culture. Regarding the specificity, both methods showed low specificity; however, the specificity of the NAT test was better than that of the conventional culture. In order to overcome this barrier and improve specificity, we need to define specific cut-offs for each pathogen and a specific clinical presentation, for instance immunosuppression / chronic lung disease such as COPD. Further studies are also needed in order to evaluate the clinical outcome in combined endpoints (clinical outcome with/without antibiotics in NAT positive / culture negative samples and vice versa). In the future, the golden standard for the diagnosis could be the combination of clinical judgment, laboratory values, radiological and molecular diagnostics to obtain a quick and objective tool for the right diagnosis irrespective of the interdisciplinary knowledge of specialists in infectious diseases, respiratory medicine or intensive care medicine.

It should be emphasised that, at present, the NAT assay does not include a specific respiratory bacterial panel but rather a broad septic panel and, therefore, it should be adapted accordingly. There are several relevant pathogens that are not included in the detection panel, such as *M. pneumonia*, *Legionella* spp. and *B. pertussis* and at the same time, airway irrelevant pathogens, such as the coagulase-negative staphylococci including *S. epidermidis*, are incorporated in the panel of the assay. It would be of great therapeutic importance to modify the detection panel of the NAT assay to include atypical bacteria, such as *Mycoplasma, Legionella*.

Among the main advantages of a specific pneumonological NAT assay is the fast delivery of the results. The use of the broad range multiplex-PCR allows rapid differentiation of the most relevant pathogens in the respiratory system within 5–8 hours. In contrast, conventional cultures of Gram-positive and Gram-negative species require markedly longer periods (3–5 and 2–3 days, respectively) for growing and determining antibiotic resistance patterns. Empirical use of antibiotic treatment will be required in most patients with suspicion of LRTID before culture results are available as a significant increase in mortality has been demonstrated for delays in antibiotic therapy as short as 4–6 hours after admission to the emergency ward [22]. While molecular diagnostics are unlikely to prevent antibiotic exposure in patients with haemodynamic instability, overwhelming systemic inflammation and signs of sepsis, it could potentially support an early de-escalation strategy, thus reducing costs, side-effects and bacterial resistance development.

Another advantage of the NAT assay is the potential identification of microorganisms in patients already receiving antibiotic therapy (which would preclude bacterial growth). The later advantage was proved to be beneficial in two cases that received previous antibiotic treatment and were detected positive for *Streptococcus pneumonia* only by the NAT assay but not by the conventional bacterial culture.

However, these are two sides of the same coin. Antimicrobial resistance, which is a very important parameter governing the choice of the most effective antimicrobial treatment, cannot be tested by use of NAT. Resistance patterns for BAL pathogens is necessary to provide critical information for more successful and less harmful treatment of patients.

At present, despite the advantages of the NAT assay, which may provide rapid, reliable, accurate and cost-effective results, there is a great need to improve the sensitivity and especially the specificity of the assay by adjusting the threshold for different pathogens and for different groups of patients. Unfortunately, samples derived from the respiratory tract are not sterile owing to pre-existing commensals and potential pathogens causing colonisation, and this may lead one to overdiagnose infections. In a recent study, Schaub et al. investigated the value of multiplex real-time PCR for the early diagnosis of sepsis in the emergency department in comparison with regular blood cultures. The diagnostic accuracy of multiplex-PCR for the causative organisms of sepsis was not significantly different from the blood cultures; however, there was a decrease of 4 hours in the time delay between the initiation of the diagnostic test and the definite results [24].

In clinical practice, there is always a dilemma regarding how to treat patients on previous antibiotic therapy presenting negative conventional bacteriology and positive PCR. This is particularly true in nonimmunosuppressed patients. We believe that if the molecular diagnostic methods for the BAL can be successfully established and the required cut-off for each bacterium, depending on pathogenicity, can be defined, a clinical intervention study will be necessary to address this particular question. It would be challenging to define a reliable clinical endpoint to identify the need for antibiotic therapy in these cases. Nevertheless, the first step is to evaluate the feasibility and diagnostic properties of

this method, as compared with clinical judgment and conventional bacteriology.

The limitation of our pilot study using a septic-NAT-panel to test BAL is the small number of samples and the non-specific NAT for the respiratory system. Furthermore, it has to be noted that in clinical practice the separation of infected from noninfected lower respiratory tract is challenging because of a wide range of comorbidities, for example COPD/asthma, immunosuppression and colonisation. To distinguish LRDTI/non-LRTDI clinical judgment alone is not a promising and safe method. The combination of clinical judgment and molecular diagnostics has to be discussed for the future.

The great advantage of our pilot study is the integration of clinical assessment combined with radiology, cytological data and microbiological data based on molecular procedures (NAT) and conventional bacterial cultures. However, it seems imperative that results of bronchoalveolar bacterial NAT are interpreted in a clinical context. In this sense, we suggest that different cut-off limits for determining whether the finding is responsible for infection need to be defined for (a) immunosuppressed patients, (b) those with previous or known lung affections such as COPD and asthma; and (c) those without any previous history of lung disease. Although such discriminating cut-off limits are clinically desired, a first step in this direction could be semi-quantitative reporting of the pathogen genome load to examine its correlation with infectious disease.

Therefore, further studies on other patient populations (no previous lung pathology, known lung disease and immunosuppressed patients) and different clinical settings (on/off antibiotics, pneumonia, COPD exacerbation) are required for better definition of respective cut-off limits in such comorbidities. Finally, although a targeted adjustment of antibiotics coverage is conceptually achievable by accelerating specific bacterial diagnosis, the clinical and financial impact of these approaches needs to be evaluated in randomised controlled trials.

In conclusion, the data presented in this study indicate that the PCR- and microarray-based rapid molecular diagnostic assay offers a relative advantage to conventional cultures for detection of potentially pathogenic bacteria in bronchoalveolar lavage of patients with pneumonia.

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Disclosure statement: MK and MM are full time employees of Mobidiag Ltd, Espoo, Finland, the producer of the assay utilised in the study. All other authors declare that they have no competing interest.

Authors contribution: KJ collected and analysed the data and drafted the manuscript. MK and MM carried out rapid broad-range PCR and microarray-based nucleic acid amplification testing (NAT) assay. PG collected data. MT participated in the study design and coordination. HH provided support on data interpretation. DS conceived the study, designed and coordinated data collection, performed statistical analyses, data interpretation and wrote the manuscript. EP provided intellectual support for the manuscript. All authors provided substantial input for the design and/or realisation of the study, and read and approved the final manuscript.

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