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Diagnostic performance of multiplex PCR on pulmonary samples versus nasopharyngeal aspirates in community-acquired severe lower respiratory tract infections

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ABSTRACT

Background: PCR-based techniques for the diagnosis of community- acquired severe lower respiratory tract infections are becoming the standard of care. However, their relative ability to identify either atypical bacteria or viruses that cause LRTI from clinical samples from various sources is yet to be determined.
Objectives and study design: The aim of our study was to compare the diagnostic yield of nasopharyngeal aspirates with that of pulmonary samples for the etiological diagnosis of severe acute lower respiratory tract infections by multiplex PCR. Patients were adults with community-acquired pneumonia or acute exacerbation of chronic obstructive pulmonary disease.
Results: We obtained concordant results for 81 (79%) of the 103 pairs of samples. In 14 of the 22 discordant results, more pathogens were evidenced in the lower respiratory tract samples.
Conclusions: Pulmonary samples had a similar diagnostic sensitivity for virus detection by multiplex PCR as nasopharyngeal aspirates. In contrast, in our study, the diagnostic efficacy of pulmonary samples for *Legionella pneumophila* over simple aspirates was clearly superior.

1. Background

Acute lower respiratory tract infections (LRTI) are a major cause of morbidity and mortality worldwide. The identification of causative agents in severe community-acquired LRTI is mandatory for efficient clinical monitoring and treatment as clinical signs are poor etiological indicators. Until recently, two laboratory approaches have been used: one to identify bacterial infections from various pulmonary samples such as sputum, endotracheal aspirates, or bronchoalveolar lavage and one to identify respiratory viruses from nasopharyngeal aspirates, using generally low sensitivity assays, such as viral culture or antigen detection.

The development of multiplex real-time polymerase-chain reaction (PCR), to identify panels of viral pathogens and prevalent atypical respiratory bacteria has revolutionized the microbiological diagnosis of LRTI [1] and expanded the range of pathogens that can be identified, including a large variety of viral agents [2]. Several combinations of primers and probes are used by different manufacturers and commercial assays differ in their performances for each pathogen or group of pathogens [3,4]. Validation of the method is not easy since multiplexing may hamper the performance of amplification as compared to single PCR [5]. However, multiplex PCR have a better diagnostic yield for viruses than standard cell cultures with a specificity equal to or above 90% [6].

PCR may prove usefull for bacteria that are difficult or take a long time to culture [1]. Thus numerous multiplex-PCR assays combine detection of viruses with that of a panel of fastidious bacteria in one run with a single clinical sample. However, their relative ability to identify either atypical bacteria or viruses that cause LRTI in various clinical samples of the upper or lower respiratory tract is yet to be determined.

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2. Objectives

The aim of our study was to compare the diagnostic yield of nasopharyngeal aspirates with that of pulmonary samples for the etiological diagnosis of severe LRTI in adults hospitalised in the intensive care unit at the University Hospital of Tours.

3. Study Design

3.1. Patients and samples

We enrolled a continuous series of adults between the ages of 20 and 89 years during their first two days following admission to the intensive care unit of the University Hospital of Tours for severe community-acquired pneumonia (CAP) or acute exacerbation of chronic obstructive pulmonary disease (COPD). Patients were recruited from January 2013 to January 2014. CAP was defined as the presence of a new pulmonary infiltrate by chest radiography associated with at least one of the following clinical symptoms: fever ($> 38\,^{\circ}C$) or hypothermia ($< 35\,^{\circ}C$), cough with or without sputum, shortness of breath, or crackling sounds on lung auscultation. Exacerbation of COPD was defined as symptomatic respiratory deterioration with an arterial pH < 7.35 or the need of oxygen therapy of over 3 L/min (Table 1).

Both nasopharyngeal aspirates (NPAs) and lower respiratory tract (LRT) samples were collected for each patient at the time of ICU admission. For NPAs, a disposable catheter connected to a mucus extractor was inserted into one nostril to a depth 5–7 cm and drawn back while applying gentle suction. LRT samples consisted of induced sputum, endotracheal aspirates, or bronchoalveolar lavage, depending on the clinical status of the patient. They were initially sent to the laboratory for bacterial culture. The intended time frame berween collections was a maximum of 3 days.

The quality of all sputum and endotracheal samples was assessed by microscopic examination according to Bartlett's cytologic criteria [8]. Samples with less than 25 squamous epithelial cells (magnification x100) were considered as representative of lower respiratory secretions. Eight samples that were not filling these criteria were discarded from the analysis.

Both **sets** of results were made available to the clinician and discrepant results discussed especially when specific treatement could be indicated for instance for influenza or *Legionnella* infections.

Table 1
Clinical characteristics of the patients.

Characteristics	No. of patients (%) *N = 103*
Demographic features	
Sex (Male/Female)	72 (70) /31(30)
Age mean (years ± SD)	61.6 ± 14.8
Age range (years)	20-89
Diagnosis	
CAP	81 (78.5)
Exacerbation of COPD	22 (21.5)
Underlying conditions	
Obesity	37 (36)
Diabetes	19 (18.5)
Heart failure	14 (13.5)
COPD	34 (33)
Chronic alcoholism	23 (22.5)
Smoking	40 (39)
Immune depression	27 (26)
Chest radiography	
New infiltrate on chest X-ray	81 (78.5)
Interstitial opacity	*10 (12,3)*
Alveolar opacity	*55 (68,0)*
Interstitial and alveolar opacity	*16 (19,8)*
Clinical feature and outcome	4.86 ± 4.9
Duration of symptoms (mean ± SD)	
Simplified index of gravity (IGS II score: mean ± SD)	40.6 ± 16
Invasive mechanical ventilation	65 (63)
Mortality	9 (8.5)

SD: standard deviation; COPD: Chronic obstructive pulmonary disease.
CAP: Community acquired pneumonia.
IGS II = Indice de gravité simplifié (Simplified gravity score) used in intensive care units was.
adapted from the Simplified Acute Physiology Score (SAPS II) as defined by Le Gall et al. [7].

3.2. Ethics statement

This was an observational and non-interventional study as no additional sampling was performed. Each patient or their legal representative received a written information letter of non-opposition. The study was approved by the ethics committee of the French society of intensive care, called SRLF for "Société de Réanimation de langue Française". Clinical and biological data were stored in an anonymized database.

3.3. Detection of respiratory pathogens by multiplex PCR

3.3.1. Nucleic acid extraction

Samples were pre-treated to avoid problems linked to viscosity and prevent non-specific inhibition of the PCR reaction. NPAs were diluted with an equal volume of sterile isotonic saline solution. Sputum and endotracheal aspirates were pre-treated with an equal volume of Digest-EUR™ (Eurobio, France) for 15 min at room temperature and then centrifuged for 15 min at 1500 *g*. The supernatant was discarded and replaced by an equivalent volume of sterile water before vortexing. Both **types** of samples were then frozen until use.

DNA and RNA were extracted using an EZ1 Advanced XL automatic extractor (Qiagen, Germany). Nucleic acids were extracted from 200 µL pre-treated samples and eluted in a final volume of 90 µL.

3.3.2. Multiplex PCR

All samples were analysed using the CE-marked multiplex molecular assay RespiFinder®SMART 22 (Pathofinder, The Netherlands). This assay is based on a multiplex ligation-dependent probe amplification technique [6]. It allows the simultaneous qualitative detection of four bacteria (*Legionella pneumophila*, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae,* and *Bordetella pertussis*) and 18 respiratory viruses: influenza A, B, and A-H1N1pdm2009 virus, respiratory syncytial virus A and B, parainfluenza virus 1–4, coronavirus OC43, 229E, NL63, and HKU1, rhinovirus/enterovirus, human metapneumovirus, Adenovirus, and human bocavirus. Assay manufacturer recommandations indicate that "*a variety of specimen was suitable for the diagnosis of viral and/or bacterial infections of the respiratory tract*" and a list included: *nasopharyngeal aspirates, sputum, endotracheal aspirates and bronchoalveolar lavage.*

Assays were performed on a LightCycler 480 (Roche, Switzerland), according to the manufacturer's instructions.

3.4. Statistical analysis

Categorical variables were compared by using a Chi-square or Fisher's exact test when appropriate. A p-value < 0.05 was considered as statistically significant. Frequencies, percentages, and sensitivities were manually calculated.

4. Results

103 eligible patients had available paired nasopharyngeal aspirate and pulmonary samples and were included in the study. Eighty-one patients were hospitalised for CAP and 22 for exacerbation of COPD. The primary clinical characteristics of the patients are shown in Table 1.

Table 2
Analysis of respiratory pathogen testing results on paired Nasopharyngeal aspirate (NPA) and lower respiratory tract samples (LRT).

Results	No of samples (%)
Concordant results	81 (79)
Concordant positive results[a]	42
Concordant negative results	39
Discordant results	22 (21)
NPA > LRT	8
NPA + / LRT -	*4*[b]
NPA ++ / LRT +- or –[*]	*4*[c]
LRT > NPA	14
NPA - / LRT +	*7*[d]
NPA +- / LRT ++[*]	*7*[e]

[a] 39 infections with one pathogen and 3 co-infections with 2 pathogens.
[b] 1 rhino/enterovirus, 1 adenovirus, and 2 parainfluenza viruses (1 and 3).
[*] co-infection with 2 pathogens of which at least one was discordant between the 2 samples.
[c] 3 bocavirus type 1 and 1 co-infection with *Mycoplasma pneumonia* and parainfluenza virus 3.
[d] 1 Influenza A H1N1 pdm09 virus, 1 rhino/enterovirus, 1 adenovirus, and 4 *Legionella pneumophila*.
[e] 1 Influenza B virus, 1 coronavirus OC43 and 2 coronavirus 229E, 1 adenovirus, 1 bocavirus type 1, and 1 *Legionella pneumophila*.

Pulmonary samples consisted of 37 induced sputum, 59 endotracheal aspirates, and seven BALs analysed together and grouped under the denomination LRT for "lower respiratory tract samples". The median time between NPA and LRT sample collection was 0.5 days (range, 0–3 days where 0 indicates that the two samples were collected on the same day).

We obtained concordant results for 81 (79%) of the 103 pairs of samples, consisting of 42 positive and 39 negative results (Table 2). The results differed for 22 paired samples. Discordance is defined by the identification of a given respiratory pathogen in one paired sample, but not the other. In the presence of a co-infection with two pathogens, discordance indicates that one of the two pathogens, or even both, was (were) not identified in one of the two samples. In 14 of the 22 discordant results, more pathogens were evidenced in the LRT samples (Table A1).

We identified at least one respiratory pathogen in 64 of the 103 patients (62.1%): 50 single infections and 14 dual infections for a total of 78 pathogens. There was no difference in the prevalence of respiratory pathogens between patients with an underlying chronic respiratory disease and those without.

We detected no *Chlamydophila pneumoniae, Bordetella pertussis*, or parainfluenza virus 2 or 4 among the 18 viruses and 4 bacteria investigated by the assay. Among the NPAs, 57 (55.3%) were positive: 50 for one pathogen, and 7 for two. Among the LRT samples, 59 (57.2%) were positive: 49 for one pathogen and 10 for two.

We identified 55 of the 78 detected pathogens (70.5%) in both types of samples, nine (11.5%) only in NPAs, and 14 (18%) only in LRT samples, corresponding to the 22 discordant paired samples (Table 3). The percentage of positive samples in NPAs and LRT were comparable: 82% and 88.5%, respectively ($p = 0.36$). We identified metapneumovirus and respiratory syncytial virus in both NPAs and LRT samples for all positive cases. Three of six (50%) parainfluenza virus infections and three of five (60%) bocavirus type 1 were only positive in NPAs. In contrast, two of 29 influenza virus infections were detected only in LRT samples. There were no significant differences between detection rates of NPAs and LRT samples for any respiratory viruses. In contrast, we detected only one of the six *Legionella pneumophila* infections of our study in NPAs (16%), whereas they were all detected in LRT samples.

5. Discussion

Sampling methods (aspirates versus washes or swabs) and their localisation (nasal, nasopharyngeal, oropharyngeal, or pulmonary) for the optimal etiologic diagnosis of LRTI are still a subject of debate. Assessments of sampling methods have been hampered in many studies by limiting factors, such as the type of population (children versus adults), underlying conditions of the patients (immune competent versus immune compromised), the type of respiratory disease (upper or lower respiratory tract infections), or the spectrum of detected respiratory pathogens (bacteria versus viruses) [9].

Here, we investigated the diagnostic efficacy of nasopharyngeal aspirates versus pulmonary samples in a well-defined adult population of community-acquired severed LRTI and found that both diagnostic approaches gave similar diagnostic yield for respiratory viruses. This is discordant with several studies that have reported better performance for LRT samples for the diagnosis of viral infections in adults [10,11]. These studies where based on nasal, throat, or nasopharyngeal swabs. We used NPAs, which have been shown to provide a better yield than swabs, even in adults [12]. Accurate comparisons must also consider the quality of the so-called pulmonary samples when dealing with sputum, as contamination with saliva may skew the results. The assessment of the pulmonary origin of sputum was not documented in many studies. In our study, we assessed the quality of our pulmonary samples and found that 73% of sputum and endotracheal aspirates were *bona fide* pulmonary samples.

The superiority of lower over upper respiratory tract samples for the diagnosis of severe infuenza has been suggested by several authors [11,13]. We did not see such difference in our study, since 27 of 29 influenza virus infections were diagnosed in both specimens.

The use of molecular detection has greatly improved the diagnosis of the four atypical bacteria tested, for which culture requiring specific growth media is slow and fastidious, especially since antibiotic susceptibility is not routinely required for these bacteria [14]. Legionnaires' disease affect 10–15 CAP cases admitted to our ICU every year. Given the severity of this disease, a rapid and reliable diagnosis is needed to adapt clinical management. The detection of urinary antigen of L. *pneumophila* serotype 1 is very useful in the diagnosis, but not sufficiently sensitive. In our study, one case of Legionellosis gave a negative result for urinary antigen testing but was positive by PCR on the LRT sample. Our comparative study of NPAs versus LRT samples highlights the clear superiority of pulmonary samples for the diagnosis of L. *pneumophila* infection, as five of the six L.*pneumophila* were detected only in pulmonary samples. This result is in accordance with other studies indicating that throat or nasopharyngeal swabs are not reliable for the molecular detection of Legionella [15]. Clinicians should be aware that the absence of detection of *Legionella pneumophila* by PCR on a nasopharyngeal specimen does not exclude Legionellosis and that PCR on a LRT sample should be performed for the definitive diagnosis.

We found that LRT specimens had a similar diagnostic sensitivity for virus detection by multiplex PCR as NPAs. In contrast, the diagnostic efficacy of pulmonary samples for *Legionella pneumophila* over NPAs was clearly superior. Some authors have suggested that sputums were even preferable for the molecular detection of viruses [11,13]. However, sampling methods need to be standardised to obtain LRT specimens of good quality, in terms of contamination with saliva, along with pretreatment procedures before extraction.

Development of accurate point-of-care tests for respiratory viruses has been listed as a priority by World Health Organization to remplace empirical antibimicrobial use and thus prevent emergence of resistance. Syndromic approaches in laboratory assays has driven the development of multiplex. PCR-based techniques that associate the detection of a large panel of viruses along with several atypical bacteria. PCR-based techniques can be performed in a few hours and are much more sensitive than previous assays based on antigenic detection. In many

Table 3
Distribution of respiratory pathogens detected by multiplex PCR from nasopharyngeal aspirate (NPA) and/or lower respiratory tract samples (LRT).

	No. detected (%)				Sensitivity[a] (No. of positive results/ tot[a]l No. detected, %)		
	Total (NPA and/or LRT)	Both on NPA and LRT	Only on NPA	Only on LRT	NPA	LRT	*p* value[b]
Influenza virus (A, B, and A H1N1 pdm09)	29	27 (93)	0	2	93	100	0.49
Rhino/enterovirus	10	8 (80)	1	1	90	90	NA
Corona virus (HKU1, OC43, NL63, and 229E)	8	5 (62.5)	0	3	62.5	100	0.2
Parainfluenza virus 1 and 3	6	3 (50)	3	0	100	50	0.181
Respiratory syncytial virus A and B	5	5 (100)	0	0	100	100	NA
Human metapneumovirus	5	5 (100)	0	0	100	100	NA
Bocavirus type 1	5	1 (20)	3	1	80	40	0.523
Adenovirus	3	0 (NA)	1	2	NA	NA	NA
Legionella pneumophila	6	1 (17)	0	5	16.5	100	**0.015**
Mycoplasma pneumoniae	1	0 (NA)	1	0	NA	NA	NA
Total	78	55 (70.5)	9 (11.5)	14 (18)	82	88.5	0.366

NA: Not applicable.
[a] Sensitivity was calculated based on the presence of a respiratory pathogen in either NPAs or LRT samples over the total number of pathogens identified in both sample types.
[b] Fisher's exact test or Chi-square test.

instance, there are no specific recommendations pertaining to the type of samples to be used with such assays, although it appears that upper and lower respiratory tract samples perform differently for some pathogens.

In the same way that we have highlighted the superiority of pulmonary samples for the detection of L. *pneumophila*, comparable studies are needed to define the optimal sample and the detection limits for each respiratory pathogen of interest in LRTI. This should help to refine existing panels of respiratory pathogens, based on the type of patients and samples, and improve etiologic diagnosis.

Author contributions

SR conducted the viral experiments with the help of CLB. CL coordinated the collection of clincical specimens with the help of AL and JM. SR ans CL contributed equally to the analysis.

DG and AG initiated the study, planned the experimental design and contributed to write the paper. All authors read and approved the final manuscript.

Conflict of interest

None.

Funding

None.

Ethical approval

This was an observational and non-interventional study as no additional sampling was performed. Each patient or their legal representative received a written information letter of non-opposition. The study was approved by the ethics committee of the French society of intensive care, called SRLF for "Société de Réanimation de langue Française". Clinical and biological data were stored in an anonymized database.

Randomized controlled trial

NA.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2018.08.001.

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