THE IMPORTANCE OF SPUTUM SAMPLES IN FINDING A VIRAL AETIOLOGY OF PNEUMONIA

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ABSTRACT

Background: For detection of respiratory viruses and establishing their primary causative role in patients with community-acquired pneumonia (CAP), samples from the lower respiratory tract seem to have obvious advantages over conventional upper respiratory tract samples, because they originate from the site of infection. Nevertheless, molecular diagnostics are complicated by the viscous character of sputum. In this study, we aimed to assess the value of viral molecular diagnostics on sputum samples, used to establish the aetiology of CAP in addition to conventional diagnostics.

Methods: The influence of storage and the way of processing of sputum on the sensitivity of reverse transcriptase-PCR (RT-PCR) reactions to detect respiratory viruses was investigated in two subsequent experiments. Sputum specimens for these optimisation experiments were obtained from patients hospitalised for CAP in the St Antonius Hospital (Nieuwegein, the Netherlands). Subsequently, we tested sputum samples from patients with CAP, admitted to the St Antonius Hospital (Nieuwegein, the Netherlands) and the Gelderse Vallei Hospital (Ede, the Netherlands), for the presence of respiratory viruses and specific bacteria.

Results: Storage of sputum samples at -80°C did not influence the yield of viral PCR. The magnalyser method enabled the extraction of RNA necessary for PCR in both low and high viscosity sputum without measurable damaging the viral RNA. Despite the use of a wide range of microbiological techniques and clinical materials (blood culture, sputum culture, urine antigen test, serology, nose/throat swab PCR, sputum PCR), in about 40% (120) of the CAP patients no pathogen associated with CAP could be detected. For a total of 58 patients, paired sputum and nose-throat samples were available. PCR on sputum samples resulted in reduced diagnostic deficit compared to the PCR on combined nose and throat swabs.

Discussion: Viral molecular diagnostics on sputum samples can lead to an enhanced detection of respiratory viruses in CAP patients. Particularly in cases where diagnostics are essential to guide control measures, sputum PCR can be an important addition, for both individual patient management and the public health.

INTRODUCTION

Worldwide, community-acquired pneumonia (CAP) remains an important cause of morbidity and mortality. Common pathogens associated with CAP are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, respiratory syncytial virus (RSV) and influenza viruses [1-6]. Conventional diagnostics available for patients with CAP, including sputum culture, blood culture, urine antigen tests on *Streptococcus pneumoniae* and *Legionella pneumophila* and detection of antibodies in serum, mainly focus on respiratory bacteria [7,8]. Recent developments in molecular diagnostics resulted in improved sensitivity for the detection of micro organisms, especially viruses, that are fastidious, no longer viable or present in small amounts [8,9]. Adequate detection of viruses can be important for the implementation of control measures in case of outbreaks.

Aetiological studies, using polymerase chain reaction (PCR) in addition to conventional diagnostics, indicate that virus-associated CAP is frequently seen in adults [10-13]. These findings are mainly based on conventional samples for virus diagnostics taken from the upper respiratory tract, like nose swabs and throat swabs [10-13]. Nevertheless, it is unclear whether viruses detected in the upper respiratory tract, represent the cause of infection or a coincidental upper respiratory tract infection (RTI) [14]. Moreover, recent studies utilising PCR show the detection of respiratory pathogens in upper respiratory tract specimens collected from persons without respiratory complaints [15-18].

For detection of respiratory viruses and establishing their primary causative role in patients with CAP, samples from the lower respiratory tract seem to have obvious advantages, because they come from the site of infection. Sputum is the least invasive lower respiratory tract sample to obtain. Nevertheless, molecular diagnostics on sputum are complicated by its viscous character, and the presence of PCR-inhibiting factors [19]. The viscosity of sputum leads to heterogeneous samples, in which micro organisms are unevenly distributed, making the extraction of nucleid acids impossible. This might result in false-negative results, and is therefore a complicating factor for the reproducibility of molecular diagnostics [20, 21]. To handle these problems, sputum samples need to be liquefied before molecular virus detection can be performed.

In this study, we aimed to assess the value of viral molecular diagnostics on sputum samples used to establish the aetiology of CAP. Therefore, we studied

the influence of sputum storage at -80°C, and two methods for sputum treatment, before nucleic acid isolation, on the sensitivity of PCR to detect respiratory viruses in sputum specimens. Subsequently, we tested sputum samples from patients hospitalised with CAP. Diagnostics were performed using the PCR on sputum samples in addition to PCR on matched nose and throat swabs and conventional microbiological techniques.

METHODS

SPUTUM SPECIMEN HANDLING

The influence of storage and processing of sputum on the sensitivity of reverse transcriptase-PCR (RT-PCR) reactions to detect respiratory viruses was investigated in two subsequent experiments (table 1). The viruses used for RT-PCR within these experiments, influenza virus A(H3N2) and respiratory syncytial virus (RSV) type A, were propagated in tertiary monkey kidney cells. As internal control for specimen processing and RT-PCR equine arteritis virus (EAV) propagated in Baby Hamster Kidney cells was used. Sputum specimens for these optimisation experiments were obtained from patients hospitalised with CAP in the St Antonius Hospital (Nieuwegein, the Netherlands).

Table 1. Experiments studying the influence of sputum specimen handling on the performance of viral RT-PCR.

Experiment	Origin sputum	Spiked viruses	Storage	Processing	RNA isolation
1	Pooled sputum of 11 persons	Influenza A RSV A EAV[1]	No storage versus storage for 2 or 4 weeks at -80°C	Sputolysin	Roche viral nucleic acid kit
2	10 sputum samples	Influenza A RSV A	-80°C	Magnalyser versus Sputolysine	Roche viral nucleic acid kit

[1] internal PCR control

SPUTUM SAMPLE PREPARATION

After collection, the sputum samples were stored at -80°C. In the first experiment, the influence of (duration of) storage of sputum at -80°C, prior to virus detection using PCR, was investigated. Sputum from 11 patients was pooled, divided in portions of approximately 2 ml, and subsequently lysed with sputolysine. Therefore, 6 ml of sputolysine (Calbiochem, NJ, USA) was added to the pooled sputum and vigorously vortexed. After end-over-end rotating for 30 minutes at ambient temperature, the mixture was neutralised with 0.88 ml 100 mM tris/10mM EDTA pH 8.0 and centrifuged for 5 minutes at 1,250 rpm. The supernatant was then aliquoted in 200 µl proportions. Subsequently, 200 µl lysed sputum was spiked with 40 µl virus mixture containing influenza virus A(H3N2) and 40 µl RSV type A. After spiking, samples were either directly subjected to RNA isolation (t=0), or stored at -80°C for 2 and 4 weeks, and subsequently subjected for nucleid acid isolation. As positive control, 40 µl PBS with 120 µl sputolysine, and 40 µl of virus mixture identical to the samples was used. As negative control, 200 µl sputum with 40 µl PBS was used. EAV was used as internal PCR control. Twenty µl EAV of standard used concentration was added just before RNA isolation.

PROCESSING SPUTUM SAMPLES

In the second experiment, two common methods to lyse samples, namely sputolysin treatment and magnalyser treatment (Roche, Almere, the Netherlands), were compared. For this purpose, 10 sputa were aliquoted in two portions of 50-100 µl and both spiked with 50 µl spike mixture containing influenza virus A(H3N2) and RSV type A. The first aliquot was lysed with sputolysine preheated to 37°C for 15 minutes. After sputolysine treatment, 400 µl lysisbuffer from the manual viral RNA extraction kit (Roche, Almere, the Netherlands) was added and the samples were rotated end-over-end for 2 hours at room temperature. The second aliquot was mixed with 400 µl lysisbuffer and transferred to magnalyser greenbead tube (Roche, Almere, the Netherlands) and beated 30 seconds at 6,000 rpm. After the magnalysing and sputolysine treatment samples where cooled at 4°C and spun for 1 minute at 14.000 rpm. The lysed sputum was transferred to a fresh microtube and further analysed. As positive control 40 µl PBS, 120 µl sputolysine and 40 µl of virus mixture identical to the samples were added. As negative controls, unspiked sputum and PBS were used. All samples were tested non-diluted, as well as 10 times and 100 times diluted.

MICROBIOLOGICAL AETIOLOGY OF CAP

In addition to conventional diagnostics [22], the optimal method for sputum handling with respect to storage and pre-treatment for nucleid acid isolation was applied to detect respiratory viruses and bacteria not detectable by conventional bacteria culture in sputum by PCR.

STUDY POPULATION

We included patients hospitalised in the 880-bed St Antonius Hospital in Nieuwegein or the 500-bed Gelderse Vallei Hospital in Ede in the Netherlands (both teaching hospitals) with a CAP in the period from November 2007 to September 2010, who were recruited for a randomised, double-blinded, placebo controlled trial, aiming to assess the effect of addition of dexamethasone on the length of stay in the hospital [22]. Inclusion for this trial was restricted to patients 18 years or older with a CAP requiring hospitalisation. CAP was defined as the presence of new opacities shown on the chest radiograph in combination with at least two of the following findings: cough, production of sputum, temperature >38.0 °C or <36.0 °C, audible abnormalities by chest examination compatible with pneumonia, leukocytosis (>10.000 cells/mm^3), leftward shift (>10%) or leukopenia (<4000 cells/mm^3) or CRP >15 mg/l (three times upper limit of normal). Immunocompromised patients, patients requiring intensive care unit (ICU) treatment and patients with corticosteroids intolerance were excluded.

The following data were registered as part of the study: patient characteristics (including day of birth and sex), co-morbidities and severity of the pneumonia, defined as the Pneumonia Severity Score (PSI)-score [23]. In addition to samples taken for standard patient care [22], a combined nose and throat swab as well as sputum samples were taken on the date of admission to the ER, to assess the added value of PCR in sputum samples on a panel of respiratory viruses.

Eligible patients provided written informed consent and the study was approved by the institutional Medical Ethics Committee of the St Antonius Hospital.

LABORATORY ANALYSES

Total nucleic acid was extracted with the viral nucleic acid kit (Roche, Almere, the Netherlands). Both the combined nose and throat swabs and sputum samples were tested for adenovirus, coronavirus, enterovirus, human metapneumovirus

(hMPV), influenza virus, parainfluenza virus, rhinovirus and respiratory syncytial virus (RSV), as well as for *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Chlamydophila psittaci*. PCR was performed at the National Institute of Public Health and the Environment (Bilthoven, the Netherlands) according to standardised protocols [15,24].

Bacteriological and viral cultures, PCR, urine antigen tests and serological tests were performed at the Department of Medical Microbiology and Immunology of St Antonius Hospital (Nieuwegein, the Netherlands) according to standardised protocols (enzyme immunoassay, Binax-NOW; Binax, Portland, ME, USA) [22].

STATISTICAL ANALYSES

Descriptive statistics for the study population were calculated, results of categorical variables are presented as percentages and continuous variables as median with range. Logistic regression analyses were used to examine whether the detection of bacteria was related to more severe CAP in comparison to the detection viral pathogens. The dependent variable (i.e. severe disease) was defined as a PSI-score $\geq$4.

Analyses with respect to the added value of the sputum PCR were restricted to patients for whom paired sputum and nose-throat samples were available. Statistical analyses were performed using SAS software, version 9.1.3 (SAS Institute, Cary, NC, USA).

RESULTS

SPUTUM SPECIMEN HANDLING

Complete lysis of sputum with sputolysin was very much dependent on the consistence of the sputum, and determined in this way how successful nucleid acids could be extracted. More fluid sputa were rather quickly lysed, whereas very viscous ones were not sufficiently lysed. Incomplete lysis leads to cloggy viscous heterogeneous samples. Due to the high viscosity of the sputum samples, the filter columns for RNA isolations were clogged for approximately 30%-50% of these samples, seriously hindering RNA extraction, and making diagnostics impossible. None of the filter columns were clogged following lysis of sputum by means of the

magnalyser method, as this procedure was not influenced by the consistence of sputa. Since the RNA was not measurable damaged by this method, the sputum samples could be used for RNA isolation (data not shown).

As can be seen in figure 1, the crossing points (CP) values, used to compare the relative amount of virus in samples, were similar before and after storage. This indicates that the amount of detected RNA was comparable before and after storage. The effect of diluting the samples was the same in both the sputum and PBS control samples, indicating that the PCR was not strongly inhibited by the sputum. Next to PBS, also GLY-medium was used, since the nose and throat swabs are collected in this platform. The CP values, indicating the relative amount of virus, show that the used platform (PBS, GLY or sputum) hardly influences the amount of detected RNA (figure 1).

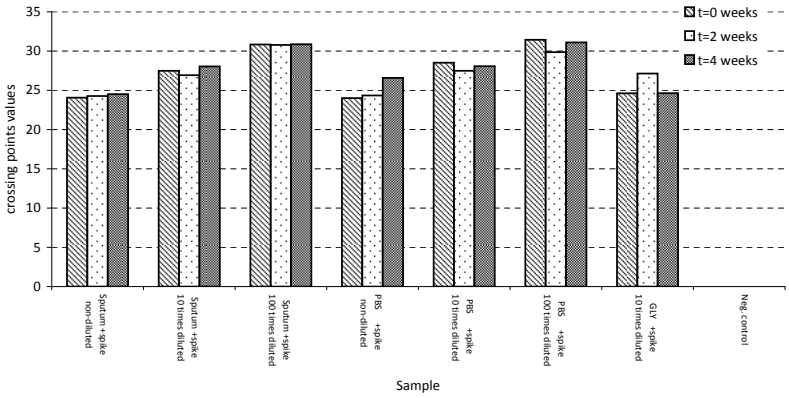

Figure 1. Recovery of respiratory syncytial virus (RSV) type A after storage at -80°C .

STUDY POPULATION

A total of 304 patients were included in the study on the aetiology on CAP. Characteristics of these patients are summarised in table 2. Co-morbidities were reported for 133 patients (44%). The PSI-score ranged from 16 to 185 points, with a median of 89.

Table 2. Baseline characteristics of patients with community-acquired pneumonia admitted to a large regional hospital in the Netherlands (N=304).

Characteristic	n	%
Sex		
male	171	56
female	133	44
Co-morbidities*		
heart failure	48	16
diabetes mellitus	43	14
chronic obstructive pulmonary disease	34	11
disease of central nervous system	30	10
kidney failure	30	10
malignancy	19	6
liver failure	2	0.7
Age (years)		
median [range]	66 [18-98]	

* Patients with multiple co-morbidities are counted for every co-morbidity

Despite the use of a wide range of microbiological techniques and clinical materials (blood culture, sputum culture, urine antigen test, serology, nose/ throat swab PCR, sputum PCR), in 120 of the CAP patients (39.5%) no pathogen associated with CAP could be detected (table 3). Only bacterial pathogens were detected in 124 patients (40.8%), only viral pathogens in 32 patients (10.5%), and both bacterial and viral pathogens in 28 patients (9.2%). There was no significant difference in the rate of severe CAP in patients for whom bacterial pathogens were detected compared to patients with viral pathogens (OR = 0.8; 95%CI: 0.4-1.8).

For a total of 58 patients, paired sputum and nose-throat samples were available. A positive PCR in sputum and/or nose-throat samples was found for a total of 22 of these patients (38%). For 14 of the 22 patients (64%) the pathogen was only detected in the sputum sample, while for four of them (18%) the pathogen was only detected in the nose-throat sample. Coronavirus, enterovirus and *Chlamydophila pneumoniae* were only detected in the sputum samples. Three of the six PCR's positive for influenza virus would have been missed, if sampling was limited to nose-throat swabs. For 11 of the 14 patients (79%) for whom respiratory viruses were detected solely in sputum, also respiratory bacteria were detected (*S.pneumoniae* (n=6), *H.influenzae* (n=4) and *C.burnetii* (n=1)). It has to be noticed that these findings must be interpreted with caution because of the low numbers.

Table 3. Number and percentage of detected pathogens in patients hospitalised because of community-acquired pneumonia (N=304).

Pathogens	n	%
Bacteria*	152	50
Viruses	60	20
Rhinovirus	22	7
Influenzavirus	11	4
Enterovirus	7	2
Parainfluenza virus	6	2
Human metapneumovirus	6	2
Respiratory syncytial virus	4	1
Coronavirus	3	1
Adenovirus	1	0.3
Epstein-Barr virus	1	0.3
No pathogens	120	39

* Bacteriological results are described in detail by Meijvis et al. [22]

Table 4. Pathogens detected in paired sputum and nose-throat samples from patients hospitalised because of community-acquired pneumonia (N=58).

	Pathogen detected in:						
	both sputum and nose/throat swab		nose/throat swab		sputum		total
Pathogen	n	%	n	%	n	%	N
Rhinovirus	1	17	1	17	4	67	6
Influenzavirus	0	-	3	50	3	50	6
Respiratory syncytial virus	1	50	0	-	1	50	2
Coronavirus	0	-	0	-	2	100	2
Enterovirus	0	-	0	-	2	100	2
Mycoplasma pneumoniae	1	50	0	-	1	50	2
Human metapneumovirus	1	100	0	-	0	-	1
Chlamydophila pneumoniae	0	-	0	-	1	100	1
total	4	18	4	18	14	64	22

DISCUSSION

Because of the viscous character, pre-treatment of sputum samples before nucleid acid extraction is essential for the good performance of PCR assays. We showed that storage of sputum samples at -80°C did not influence the virus detection. Moreover, the magnalyser method enables the RNA isolation necessary for PCR, by proper lysis of sputum samples without detectable damaging the viral RNA. Furthermore, we showed that PCR on sputum samples resulted in an increased detection of respiratory viruses and certain bacteria, compared to testing of combined nose and throat swabs only, thereby decreasing the diagnostic deficit.

In line with other aetiological studies on CAP, *S.pneumoniae* (21%) was the most frequently detected pathogen [6,11,13,25]. A remarkably high proportion of *Coxiella burnetii* (9%) was found in our study, which is probably related to the large outbreak of Q fever in the Netherlands in the period from 2007 to 2010 [26-28].

Respiratory viruses, whether or not in combination with a bacterial pathogen, were the second most commonly detected pathogens in our study (20%), as also found in other studies [6,8,9,11,13,25]. Although viral infections are common in patients with pneumonia, still little is known about the exact role of viruses. Rhinovirus and RSV are capable of invading and replicating in the lower respiratory tract mucosa [29,30], but it is unclear whether the virus infection is the primary cause of pneumonia or paves the path for a secondary bacterial infection. Sputum production is one of the symptoms of CAP. Respiratory viruses detected in sputum are likely to originate from the site of infection, i.e. the lower respiratory tract. This is most plausible when these viruses are not simultaneously detected in upper respiratory tract samples. To study the causal link between clinical disease and respiratory viruses as well as certain bacteria detected in sputum, a case-control study comparing the prevalence of respiratory viruses in the lower respiratory tract between CAP patients and an asymptomatic control group is preferred. However, this is complicated by the fact that healthy persons do not produce sputum, and other specimens of the lower respiratory tract, like bronchoalveolar lavage (BAL), are not comparable to sputum and are considered too invasive for asymptomatic persons.

Although the performance of reliable diagnostic tests in sputum is not always possible, because not all CAP patients produce sufficient sputum [31] and the quality of diagnostic tests can be influenced by the appearance of the collected sputum, varying from mucous to purulent, PCR on sputum can be a valuable addition to conventional diagnostics. Particularly in cases where regular diagnostics fail to identify pathogens, the enhanced detection of respiratory viruses and certain bacteria using PCR on sputum can have a significant meaning for patient management or public health. For example, avian influenza virus A(H5N1) can attach to the human lower respiratory tract, causing severe infections with high mortality rates [32]. The detection of highly pathogenic avian influenza viruses as cause of pneumonia in travellers or inhabitants of endemic areas is therefore essential to take immediate actions like patient isolation and contact tracing. Moreover, in outbreak settings with non-specific respiratory syndromes, identification of causative pathogens is essential to guide control activities aiming to reduce the impact of the outbreak. Background surveillance data on the prevalence of viruses in sputum will be relevant, to put the results of virus detections during an outbreak into context.

In conclusion, viral molecular diagnostics on sputum samples can lead to an enhanced detection of respiratory viruses in CAP patients, and can be substantial advantageous next to conventional diagnostics, for both individual patient management and public health.

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