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Impact on clinical decision making of quality control standards applied to sputum analysis in COPD☆

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Summary
Purpose: Sputum analysis is important in COPD exacerbation management. We determined whether application of stringent quality control criteria for sputum samples had an impact on culture results.
Methods: We analyzed sputum samples of 108 patients during stable COPD and during exacerbations. To all samples quality control standards and culture interpretation rules according to the American Society of Microbiologists (ASM) were applied.
Results: In sputum exacerbation samples considered appropriate according to ASM quality standards, criteria for infection (40%) were met more often compared to inappropriate samples (13%) ($p < 0.001$). The same pattern was observed when applying these rules to sputum samples obtained during stable disease, (50% vs. 18%, $p < 0.001$). There was no difference in the percentage of infectious cultures obtained during the stable state and exacerbations.
Conclusions: Applying stringent quality control criteria to sputum samples can have a profound effect on the labeling of sputum samples as infectious, and therefore on clinical decision making.

☆ *Statement of originality and clinical relevance*: This paper describes the importance of stringent quality control criteria on sputum samples in COPD patients. Since there is a high variability in the quality of sputum samples it is plausible that using samples of inadequate quality can have major consequences on the interpretation of culture results and therefore on clinical decisions.
Based on our results we concluded that applying stringent quality control criteria to sputum samples can have a profound effect on the number of positive sputum cultures and by inference on clinical decision making. It is therefore necessary that quality criteria are used and published in the literature on sputum outcomes in COPD. Furthermore, more longitudinal research is needed on the change in bacterial load from the stable state to exacerbations.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of chronic morbidity and mortality throughout the world. Morbidity and mortality among patients with COPD are for a large part related to acute exacerbations of COPD, which occur on average 1—3 times a year.[1] COPD exacerbations are heterogeneous events that are thought to be caused by complex interactions between the host, respiratory viruses, airway bacteria, and environmental pollution, leading to an increase in the inflammatory burden.[2] Sputum analysis is an important clinical tool in the management of COPD, especially in COPD exacerbations, for decisions to prescribe antibiotics. The most common test in exacerbations is taking a sputum sample for culture to determine the presence, abundance, and resistance pattern of bacteria. Multiple studies show that there is a variety of microorganisms present in cultures of patients with acute exacerbations of COPD.[3,4]The presence of microorganisms however does not directly signify that these organisms are the causative agent of an infection; patients can just be colonized.

In addition to culturing sputum, other clinical features and markers have been used to determine the nature of COPD exacerbations. A marker that has been commonly used since the study of Anthonisen et al. in 1987, is sputum purulence,[5] which is seen as an indirect marker for the presence of bacteria. Also Stockley et al. used sputum color (or purulence) as the primary parameter to decide whether or not to start antibiotic treatment.[6] Additionally, the concentration of inflammatory markers and cell types are also commonly analyzed in sputum samples. Sethi and colleagues showed that neutrophilic airway inflammation and systemic inflammation are more intense with well-defined bacterial exacerbations than with nonbacterial exacerbations.[7] Other parameters that can indicate the presence of a bacterial infection are C-reactive protein, TNF alpha and interleukins (IL-6, IL-8).[8—10]

All these studies show that sputum analysis is an important clinical tool in the management of COPD.

When collecting sputum samples, there is a high variability in the quality of the samples obtained. In 1975 Murray et al. designed criteria for the quality of sputum. Specimens were categorized according to the number of leukocytes and squamous epithelial cells observed microscopically in a Gram-stained smear.[11] When the number of squamous epithelial cells was far greater than the number of leucocytes in the sputum, they assumed that this specimen originated from the upper airways and therefore should not be further analyzed. The American Society of Microbiologists (ASM) also advocates quality control standards and culture interpretation rules.[12] Although these quality statements have now existed for many years, published studies involving sputum analysis often do not report on the quality control standards they used for sputum samples and whether sputum samples of inadequate quality were removed from further analyses. We hypothesize that using sputum samples of inadequate quality can suggest an active infection in the lower airways while the sputum sample originates from the pharynx and therefore this influences the interpretation of culture results and clinical decision making. Therefore, we performed a study on sputa of COPD patients in the stable state and during acute exacerbations to study the differences between adequate and inadequate sputum samples as based on the ASM criteria with regard to the incidence of infection and levels of inflammatory markers.

Methods

Patients

To be eligible for the study the patients had to meet the following criteria: (1) a clinical diagnosis of COPD, as defined by American Thoracic Society criteria [13]; (2) no history of asthma; (3) no exacerbation in the month prior to enrolment; (4) current or former smoker; (5) age between 40—75 years; (6) baseline pre-bronchodilator forced expiratory volume in one second (FEV_1) 25—85% of predicted; (7) pre-bronchodilator ratio FEV_1 to inspiratory vital capacity (IVC) 60% or less; (8) reversibility of FEV_1 post inhalation of 80 μg of ipratropium bromide via metered dose inhalator with an aerochamber $\leq$12% predicted [14]; (9) total lung capacity (TLC) greater than the TLC predicted minus 1.64 $\times$ SD; (10) no maintenance treatment of oral steroids or antibiotics; (11) no medical condition with low survival or serious psychiatric morbidity (e.g. cardiac insufficiency, alcoholism); and (12) absence of any other active lung disease (e.g. sarcoidosis).

The use of medication such as nasal corticosteroids, theophyllines, chronic use of acetylcysteine and all other bronchodilators was allowed.

The hospital's medical ethical committee approved this study. All patients provided written informed consent.

Study design

This study was performed on data of the COPE study.[15,16] Patients were instructed to contact the study personnel any time they experienced a worsening of their respiratory symptoms. They were subsequently invited to visit the outpatient department within 12 h for lung function measurements, sputum collection and consultation by one of the study physicians. Exacerbations were defined as worsening of respiratory symptoms that required treatment with a short course of oral corticosteroids or antibiotics as judged by the study physician. The stable state was defined as a period of 4 weeks with no change in respiratory symptoms beyond day-to-day variations.

Outcome measures

Sputum samples were taken in the stable state and during acute exacerbation. During the collection of the stable state sputa all patients were on inhaled corticosteroids (ICS) according to the COPE protocol. The samples obtained during exacerbation were taken both from patients on ICS and patients on placebo in line with the design of the COPE study.[16] Sputum samples were collected in sterile vials and processed in the laboratory within 4 hours. Sputa were homogenized by incubation at 37 °C for 15 min with an equal volume of 0.1% dithiothreitol.

In line with the ASM criteria, sputum samples with less than 10^5 mL^{-1} squamous epithelial cells were considered representative bronchial samples and were defined as adequate samples. All other samples were classified as inadequate.[12,17] A Gram stain and semi-quantitative culture were performed for all collected sputum samples, irrespective of whether the sample was defined as adequate. Bacterial infection was defined per ASM criteria as the presence of potential pathogenic microorganisms (PPM) in pure culture or as the presence of one or more PPM in excess (one log or more) to normal microbiological flora in sputum. Criteria for bacterial colonization were also defined also by ASM criteria as the presence of PPM in culture in equal amount or less compared to normal microbiologic flora in sputum.[12,17]

In all sputum samples IL-6, IL-8 and TNF-α concentrations were quantified using PeliKine Compact™ human sandwich ELISA kits (Sanquin, CLB, Amsterdam the Netherlands). These tests were standard tests with an inter- and intra-test variability of a set of selected reference samples, which never exceeds 10%. To process the sputum samples DTT was not used. The sputum was homogenized and analyzed without adding any agent. MPO enzymatic activity in sputum was determined by colorimetric change in absorbance during a reaction with *O*-dianisidine dihydrochloride (Sigma-Aldrich©). Spirometry was performed according to standardized guidelines.[18] FEV_1 and IVC were measured until three reproducible recordings (less than 5% difference) were obtained. The highest values were used for analyses.

Statistical analyses

Baseline characteristics are reported as mean ± SD or as numbers with corresponding percentages for categorical or dichotomous variables. Not normally distributed variables are reported as median with corresponding range.

To identify whether there were differences between sputum of adequate and inadequate samples, Chi-square or Fisher exact tests were performed as appropriate.

Results

Sputum samples of 108 patients were used for analyses. A total of 261 sputum samples were collected, of which 122 were in the stable state and 139 during acute exacerbation. Baseline characteristics of all patients are shown in Table 1.

Table 1 Baseline characteristics. Data are presented as mean (SD) or number (%).

	($N = 108$)
Age in years	64.0 (7.3)
Male	91 (84%)
Lung function post bronchodilation	
FEV_1 in L	1.68 (0.57)
FEV_1% predicted	57.4 (14.5)
IVC in L	3.8 (0.87)
FEV_1/IVC %	44.4 (10.8)
Total number of sputum samples during exacerbation	139
Total number of sputum samples during the stable state	122

Sixty of the 139 (43%) samples collected during exacerbations were classified as inadequate (Table 2). In sputum samples of adequate quality the probability of meeting the criteria for bacterial infection was increased threefold (CI 95%: 1.58−3.05; $p < 0.001$) compared to inadequate sputum samples. Without the application of the quality criteria standard the overall percentage of sputa meeting the criteria for bacterial infection was 29%, compared to 40.5% in sputum samples of adequate quality (Table 2).

Table 2 also shows the number of sputa obtained in the stable state, again divided into adequate and inadequate sputum samples. Sixty-six of the 122 (54%) collected samples were classified as inadequate. In adequate sputum samples the probability of meeting criteria for bacterial infection was increased 2.8-fold (CI 95%: 1.49−2.88; $p < 0.001$) compared to inadequate sputum samples. Without the application of the standard ASM quality criteria the overall percentage of sputa meeting the criteria for bacterial infection was 36%, compared to 50% in sputum samples of adequate quality.

In sputa obtained during the stable state 1.25 times (CI 95%: 1.01−1.56; $p = 0.04$) more sputa were classified as inadequate compared to sputa collected during exacerbation. Furthermore, the adequate sputa samples obtained during the stable state and during exacerbations showed no

Table 2 Number of sputa that met criteria for bacterial infection in adequate and inadequate sputum samples obtained during COPD exacerbation and number of sputa that met criteria for bacterial infection in adequate and inadequate sputum samples obtained in the stable state.

	Bacterial infection	No bacterial infection
Exacerbation		
Adequate sputum quality	32 (40.5%)	47 (59.5%)
Inadequate sputum quality	8 (13.3%)	52 (86.7%)
p-Value	<0.001	<0.001
Stable state		
Adequate sputum quality	28 (50.0%)	28 (50.0%)
Inadequate sputum quality	12 (18.2%)	54 (81.8%)
p-Value	<0.001	<0.001

Table 3 Concentration of inflammatory markers in adequate and inadequate sputum samples obtained during COPD exacerbation. Concentrations are displayed as median (25—75 percentiles).

	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)[a]	MPO (extinction)
Bacterial infection				
Adequate sputum	600 (179—1001[b])	7 (1.3—32)	241 (150—241[a])	1.3 (0.7—2.1)
Inadequate sputum	175 (61—453)	8.9 (0.6—58.1)	200 (16—241[a])	1.3 (0.5—1.8)
p-Value	0.044	0.753	0.130	0.787
No bacterial infection				
Adequate sputum	39 (9—725)	34 (8—100)	50 (15—241[a])	1.0 (0.1—2.1)
Inadequate sputum	13 (0.0—115)	17 (4.6—46.5)	36 (4.4—70)	0.3 (0.1—1.0)
p-Value	0.010	0.100	0.052	0.067

[a] All IL-8 concentrations >240 were labeled 241.
[b] All TNF-α concentrations >1000 pg/ml were labeled 1001.

difference in the number of sputa that met the criteria for bacterial infection ($p = 0.2$).

The concentration of TNF-α in sputum samples obtained during COPD exacerbations was significantly higher in adequate samples than in inadequate sputum samples, both in sputum samples that met criteria for bacterial infection and in those that did not (Table 3). No significant differences were found in other inflammatory markers (IL-6, IL-8, and MPO).

Table 4 shows that in sputum samples obtained in the stable state the concentration of IL-8 was significantly higher in adequate samples than in inadequate sputum samples in sputum samples that met criteria for bacterial infection. In sputum samples that did not meet criteria for bacterial infection, adequate sputum samples showed significantly higher concentrations of TNF-α and IL-6 than inadequate samples.

When we compared the concentration of inflammatory markers in adequate samples obtained in stables state and during COPD exacerbation, we observed no significant differences in the concentrations of TNF-α, IL-6, IL-8, and MPO between these samples.

Discussion

In sputum samples obtained during acute exacerbations of COPD, considered appropriate according to the ASM quality standards, criteria for bacterial infection (40%) were met more often as compared to inappropriate sputum samples (13%). Moreover, when applying these well-defined ASM criteria to sputum samples obtained during stable disease exactly the same pattern of more infections in adequate samples was observed (50% infections vs. 18%). Applying stringent quality control criteria to sputum samples can therefore have a profound effect on clinical decision-making.

This study showed that both during exacerbations and in the stable state a large number, respectively, 54% and 43% of the sputum samples were inadequate. Roche et al. also observed a low percentage of sputum samples from COPD patients (20.5%) that satisfied their quality criteria (i.e. >25 polymorphonuclear leukocytes and <10 epithelial cells per field at ×100).[19] These criteria are equal to the Murray criteria.[11] Although the study of Roche et al. was performed in COPD patients admitted to the hospital for an exacerbation and they used quality criteria that differed from the ASM criteria, both studies show that collection of inadequate sputum samples is a very common feature in COPD patients.

Our finding of so many inadequate samples is important because we observed that the criteria for infections were met considerably more frequently in adequate samples than in inadequate samples, both for samples collected during exacerbations and during the stable state. Since potential pathogenic microorganisms (PPM) causing bacterial infections have been suggested to be located in the lower airways[3] it is not surprising that samples from the upper airways, i.e. inadequate samples, meet criteria for

Table 4 Concentration of inflammatory markers in adequate and inadequate sputum samples obtained in the stable state. Concentrations are displayed as median (25—75 percentiles).

	TNF-α (pg/ml)[b]	IL-6 (pg/ml)	IL-8 (pg/ml)[a]	MPO (extinction)
Bacterial infection				
Adequate sputum	715 (220—1001[b])	18.5 (1.2—78.3)	241 (241—241[a])	1.5 (0.8—2.2)
Inadequate sputum	1001 (225—1001[b])	17.5 (3.1—77.5)	122.5 (65.8—241[a])	1.7 (0.2—2.9)
p-Value	0.588	0.376	0.009	0.651
No bacterial infection				
Adequate sputum	120 (4.5—792.5)	78.0 (15.3—388.3)	90 (9—241[a])	0.6 (0.2—2,0)
Inadequate sputum	10.3 (0.0—99.8)	19 (1.7—50)	22 (3.5—241[a])	0.2 (0.1—1.1)
p-Value	0.035	0.020	0.068	0.156

[a] All IL-8 concentrations >240 were labeled 241.
[b] All TNF-α concentrations >1000 pg/ml were labeled 1001.

bacterial infection less often. Furthermore, dilution of sputum with saliva can cause lower concentrations of PPM in the obtained samples and therefore failure to meet criteria for bacterial infection. As mentioned earlier, our study showed a higher percentage of inadequate samples collected during the stable state compared to samples collected during exacerbation. A possible explanation for this might be the difficulty of coughing up of spontaneous sputum in the stable state. A solution would be the use of sputum induction. Sputum induction has been proven to be safe in the stable state, even in patients with moderate to severe COPD[20] and recently it has also been shown to be safe in COPD exacerbations in patients with mild to moderate COPD.[21]

Many contradictory results have been published on sputum inflammatory markers in COPD.[10,22–25] In our study we also looked at sputum markers. Before discussing our results we however should mention that half of our patients received ICS. Since the literature is not consistent about the effect of ICS[26,27] on inflammatory markers associated with neutrophils, we combined the data of the groups with and without ICS in the analyses presented here.

We found that levels of TNF-α differed between adequate and inadequate samples obtained during exacerbation. In samples obtained in the stable state that met criteria for bacterial infection, the concentration of IL-8 differed between adequate and inadequate samples. In samples obtained in the stable state that did not meet criteria for bacterial infection, the concentrations of TNF-α and IL-6 differed between adequate and inadequate samples. A possible explanation for the variability in observed levels of inflammatory markers and associations with various outcomes in the literature could therefore be due to different criteria used for sputum quality.

A remarkable finding of this study was that not only a high percentage of samples collected during exacerbation met the criteria for bacterial infection, but also an almost equal percentage of samples collected during the stable state met the same criteria for bacterial infection. Additionally the concentrations of the inflammatory markers TNF-α, IL-6, IL-8 and MPO also did not differ between adequate samples obtained in the stable state and during exacerbation. This raises the question whether the criteria for bacterial infection are adequate to distinguish bacterial infection during exacerbation from bacterial colonization either during exacerbation or during the stable state. We know from literature that a large number of COPD patients are colonized, varying from 33% to 100%.[28] As in our investigation, Sethi et al. also did not find an increase in bacterial load during exacerbations in their longitudinal cohort of COPD patients.[29] This raises the question whether or not infection with bacteria caused an exacerbation or this was due to, e.g. a viral infection. Finally, it is possible that the setting of our study (out-patients with COPD who were taken off inhaled steroids) might have favoured an increase in non-infectious exacerbations relative to for instance studies in patients hospitalised for a COPD exacerbation.

In conclusion, applying stringent quality control criteria to sputum samples can have a profound effect on the percentage of positive sputum cultures and by inference on clinical decision-making.

This is probably also true for the application and analysis of other biomarkers in sputum samples. It is therefore necessary that quality criteria are used and published in study reports on sputum outcomes in COPD. Furthermore, more longitudinal research is needed on the change in bacterial load from the stable state to exacerbations.

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Conflict of interest

The authors declare that there is no conflict of interest.

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