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The role of atypical respiratory pathogens in exacerbations of chronic obstructive pulmonary disease

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Abstract

The aetiology of acute exacerbations in COPD (AECOPD) is heterogeneous and still under discussion. Serological studies have suggested that *Mycoplasma pneumoniae, Chlamydia pneumoniae*, and *Legionella pneumophila* may play a role in AECOPD.

We investigated the presence of these atypical pathogens in sputum samples in patients with stable COPD and those with AECOPD using real-time PCR. This study was part of a randomised, double blind single centre study, and a total of 248 sputum samples from 104 COPD patients were included. In total, 122 stable state sputa and 126 exacerbation sputa were tested.

Of the 122 stable state sputa, all samples were negative for *M. pneumoniae* and *C. pneumoniae* DNA, whereas one sample was positive for *Legionella* non-*pneumophila* DNA. Of the 126 exacerbation sputa, all samples were negative for *M. pneumoniae* and *C. pneumoniae* DNA, whereas one sample was positive for *Legionella* non-*pneumophila* DNA.

We investigated the possible relationship between presence of atypical pathogens in patients with stable COPD and in those with AECOPD using real-time PCR and found no indication for a role of *Legionella* spp, *C. pneumoniae* or *M. pneumoniae* in stable moderately severe COPD and in its exacerbations.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality in adults. According to the Global Burden of Disease Study, COPD is the fifth most common disease and fourth leading cause of death in the world [1]. The chronic course of this disease is frequently accompanied by acute exacerbations, characterized by an acute sustained worsening of the patient's condition from a stable state, beyond normal day-to-day variations which may warrant additional treatment [2]. Morbity and mortality in COPD patients are for the most part related to these acute exacerbations in COPD (AECOPD), which occur on average one to three times a year.

The aetiology of AECOPD is heterogeneous and still under discussion. For many years, there has been controversy whether bacteria play a role in AECOPD, and thus, whether antibiotics play a role in disease management [3,4]. Several studies have shown an association between the presence of certain bacterial species such as *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and *Haemophilus influenzae* and AECOPD [3]. However, these potential pathogenic micro-organisms (PPMO) were also present in sputa obtained from COPD patients with stable disease [5]. Apart from these bacterial PPMO it was also shown that viral infections, with a prominent role for rhinoviruses, might trigger at least one-third of AECOPD [6]. Little is known about the presence of these viral PPMO in sputa obtained from COPD patients with stable disease. The term "atypical pathogen" most commonly refers to *Mycoplasma (M.)pneumoniae, Chlamydia (C.) pneumoniae*, and *Legionella (L.)pneumophila*. The role of these bacteria in AECOPD remains unclear. Serological studies suggest that these atypical pathogens may play an important role in AECOPD [7-17]. However, the interpretation of the role of these atypical pathogens in AECOPD is not easy since these organisms are difficult to cultivate from respiratory tract specimens. Also, variability among authors exists in the reliability and interpretation of the results of serological assays.

Molecular diagnostic techniques, such as polymerase chain reaction (PCR), have become useful tools for the etiological diagnosis of lower respiratory tract infections [18]. It can detect minute amounts of nucleic acids from potentially all PPMO, does not depend on the viability of the target microbe, is probably less affected by previous antimicrobial therapy than are culture-based methods, and can provide results quickly. For patients with pneumonia molecular techniques offer distinct advantages over conventional tests for the detection of *M. pneumoniae*, *C. pneumoniae* and *Legionella* spp. [18,19].

We investigated the presence of these atypical pathogens in sputum samples in patients with stable COPD and those with AECOPD using real-time PCR.

Patients and Methods

Patients. From May 1999 through March 2000, patients were recruited from the outpatient pulmonary clinic of the Medisch Spectrum Twente, a 1150-bed teaching hospital in Enschede, the Netherlands, as described previously [5,20]. The patients met the following criteria: (i) a clinical diagnosis of stable COPD, as defined by the American Thoracic Society criteria; (ii) no history of asthma; (iii) no exacerbation in the month prior to enrolment; (iv) current or former smoker; (v) age between 40 and 75 years; (vi) a baseline prebronchodilator forced expiratory volume in 1 s (FEV_1) of 25 to 80% predicted; (vii) a prebronchodilator ratio of FEV_1 to inspiratory vital capacity value of 60% or less; (viii) reversibility value of FEV_1 postinhalation of 80 μg of ipratropium bromide via a metered dose inhalator with Aerochamber of 12% the predicted value or less; (ix) a total lung capacity that was higher than the predicted total lung capacity minus 1.64 x the standard deviation; (x) no maintenance treatment with oral steroids or antibiotics; (xi) no medical condition with a low survival rate or serious psychiatric morbidity (e.g., cardiac insufficiency or alcoholism); (xii) absence of any other active lung disease (e.g., sarcoidosis). The hospital's medical ethical committee approved this study. All patients provided written informed consent.

Study protocol. This study was part of a randomised, double blind single centre study, investigating the role of inhaled corticosteroids in COPD [20]. From this study, spontaneously expectorated sputum samples of patients were obtained at 0, 4, 7, and 10 months in stable disease and an additional sputum sample was collected at each hospital visit for an acute exacerbation of COPD. Clinically, exacerbations were defined as worsening of respiratory symptoms that made the patient contact the study office resulting in treatment by the study physician.

Sputum samples. Sputa were collected at scheduled visits to the outpatient department and in cases of exacerbation. Spontaneously expectorated sputum was collected in sterile vials and processed in the laboratory within 4 h after collection. Total sputum samples were homogenized by incubation at 37°C for 15 min with an equal volume of 0.1% dithiothreitol. Gram-stained sputum samples were examined microscopically and had to contain $<10^5$ epithelial cells per mL^{-1} (i.e., <1 epithelial cell per high-power field) to be considered as representative bronchial samples. Polymorphonuclear cell count was not one of the criteria.

DNA extraction. 200 µl of sputum was processed with the NucliSens® easyMAG™ platform, (bioMérieux, La Balme Les Grottes, France) with an elution volume of 50 µl according to the manufacturer's instructions. 5 µl of the eluate was used as template in PCR. DNA was stored at -20 °C until PCR was performed.

PCR assays. For the detection of *C. pneumoniae* an assay based on the nucleotide sequences of VD2 and VD4 of the *ompA* gene of *C. pneumoniae* was used [21]. For the detection of *Legionella* two separate assays were used, targeted at specific regions within the 5S rRNA and the *mip* gene. The primers of the first *Legionella* spp. PCR-probe assay is based on the primers described by Lindsay *et al.* [22], and detected in real-time using a TaqMan probe Leg5S [23]. The second PCR was a *L. pneumophila*-specific PCR based on the sequences of the *mip* gene [23]. For the detection of *M. pneumoniae* an assay based on the P1

adhesin gene was used [24]. In short, primer M1 (forward 5'GGT CAA TCT GGC GTG GAT CT 3') and M2 (5' TGG TAA CTG CCC CAC AAG C 3') were used to obtain a 66 bp amplicon. Real-time detection was done with a fluorescent Taqman probe (5'TCCCCC GTT GAA AAA GTG AGT GGG T' FAM). Real–time PCR for all assays was performed on a Abiprism®7900HT Sequence Detection System (Applied Biosystems, Foster City, California, USA). Results were expressed as threshold cycle values (Ct), corresponding to the cycle at which PCR enters the exponential phase; Ct values are proportional to the negative logarithm of the initial amount of input cDNA. If no increase in fluorescent signal is observed after 50 cycles, the sample is assumed to be negative.

Results

104 patients (median age 63 years; range 45-75 years; 86 men, 18 women) provided a total of 248 sputum samples. 122 samples were obtained during stable disease and 126 sputa were obtained during AECOPD. 76 patients provided both stable state and AECOPD samples, 18 patients provided stable state samples only, and 10 patients provided AECOPD samples only. Because a substantial percentage of patients were unable to spontaneously expectorate an adequate sputum sample, and because we only used microscopically representive sputum samples, the number of stable state sputa is lower than theoretically expected. Of the 122 stable state sputa, all samples were negative for *M. pneumoniae* and *C. pneumoniae* DNA, whereas one sample was positive for *Legionella* non-*pneumophila* DNA (positive in 5S rRNA based PCR, negative in *mip* gene based PCR, Ct value 40). Of the 126 exacerbation sputa, all samples were negative for *M. pneumoniae* and *C. pneumoniae* DNA, whereas one sample was positive for *Legionella* non-*pneumophila* DNA (positive in 5S rRNA based PCR [Ct value 43], negative in *mip* gene based PCR). The *Legionella* positive samples were obtained from

different patients. In both *Legionella* positive samples *S. pneumoniae* was cultured a level of growth of $>10^5$ cfu/ml.

Discussion

We investigated the possible relationship between presence of atypical pathogens in patients with stable COPD and in those with AECOPD using real-time PCR and found no indication for a role of *Legionella* spp., *C. pneumoniae* or *M. pneumoniae* in COPD. Several potential contributions of bacterial infection to the aetiology, pathogenesis, and clinical course of COPD can be identified. With regards to microbial patterns and their possible involvement in the aetiology of AECOPD, it is a common view that *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhali*s are the leading pathogens. Viruses have also been shown to cause acute exacerbations, frequently working as co-pathogens together with bacterial pathogens [3,25]. Recently, extensions of this concept have been provided. In several studies serological evidence of *C. pneumoniae*, *Legionella* spp. and *M. pneumoniae* playing a role as a pathogen or co-pathogen in acute exacerbations has been reported [7-17]. One of the major issues not addressed in any of the seroepidemiologic studies is the correlation of serology with infection as defined either by isolation of the organism in culture or by PCR. The lack of standardization for performance of serological test has made the interpretation of these published data from different laboratories in COPD patients difficult and, in our view, debatable.

M. pneumoniae is a common cause of community-acquired pneumonia [2]. Lieberman *et al.* found *M. pneumoniae* to be the cause of AECOPD in 34 out of 240 (14%) hospitalised patients [7], higher than other reports about the microbiological aetiology in AECOPD. In 24 of these 34 patients (71%), there was serological evidence of infection with at least one other respiratory agent in addition to *M. pneumoniae.* Patients that received antibiotics against *M.*

pneumoniae did not have a better outcome in terms of a shorter hospital stay. The trend was actually in the opposite direction,the mean length of hospitalisation for AECOPD was 1.2 days less (5.3 versus 4.2 days). In three older studies, *M. pneumoniae* as possible aetiology in AECOPD was identified in 0.5% - 2.4% of AECOPD [26-28]. In a more recent study, Soler *et al.* did not find a single case of infection due to *M. pneumoniae* in a group of patients with severe COPD [29]. *M. pneumoniae* is a fastidious organism, and culture is time-consuming, and lacks sensitivity. Consequently, the laboratory diagnosis of *M. pneumoniae* infection has largely relied on commercially available serological tests, most often enzyme immunoassays (EIAs), microparticle agglutination assyas (MAG) and the complement fixation test (CFT). However, few of these commercial assays have appropriate performances in terms of sensitivity and specificity, and therefore some authors consider nucleic acid amplification the preferred diagnostic procedure for the diagnosis of *M. pneumoniae* infections [30].

In a study by Lieberman *et al.*, 17% of hospital admissions for AECOPD showed serological evidence of acute infection with *Legionella* spp. [9]. Our findings are in concordance with those found by Soler *et al.* who found no serological evidence for infection with *L. pneumophila* in a group of patients with severe COPD [29]. An exhaustive review of AECOPD studies that have focused on the search of an aetiological agent by means of invasive procedures, such as bronchoscopic techniques, shows that *Legionella* spp. have never been identified using culture [2]. A disadvantage of serological testing is the inability to accurately detect all *Legionella* spp. and serogroups. Although seroconversion to *L. pneumophila* serogroup 1 is generally regarded as being highly diagnostic, the sensitivity and specificity of seroconversion to other species and serogroups has not been rigorously confirmed [19,31]. *Legionella* colonization in COPD patients has never been detected and does not seem to be a risk factor for exacerbations in these patients. Of all pneumonia pathogens, *Legionella* non-*pneumophila* spp. probably presents the greatest risk for

contamination measured in PCR, given the organism's ubiquitous environmental presence [18]. Because of the high Ct values observed in both samples, amplification and detection of environmental *Legionella* DNA cannot be completely excluded.

C. pneumoniae has been reported to cause between 4% -16% of AECOPD, an observation based almost solely on serological evidence [14-17]. Studies of *C. pneumoniae* in COPD are complicated by several observations. Use of cell culture for detection of *C. pneumoniae* is technically demanding and time-consuming, and cell cultures generally have a low yield. As a consequence, the diagnosis of *C. pneumoniae* infection largely relies on serological testing using microimmunofluorescence (MIF). The pitfalls in *C. pneumoniae* serological testing are well known. Reports from different laboratories are highly variable and adequate evaluations compared to a "gold standard" are lacking, which had led for calls for more standardized approaches in diagnostic testing [32]. Background rates of seropositivity by MIF can also be very high in some adult populations, sometimes exceeding 80% [33]. In addition, smoking is associated with increased levels of serum antibodies to *C. pneumoniae* in patients with and without COPD and serological conversion occurs even in the absence of symptoms [2, 21]. In 1988 it was reported that patients with coronary artery disease carry significantly more anti *C. pneumoniae* immunoglobulin G (IgG) and IgA antibodies in their bloodstream than healthy controls [34]. Although initial reports were positive, the later ones, often prospectively designed and adjusted for known cardiovascular risk factors, showed a negative or weak positive association overall between seropositivity for *C. pneumoniae* and atherosclerosis. Methodology has a strong impact on the possible association between *C. pneumoniae* and atherosclerosis: detection of the link between *C. pneumoniae* and coronary artery disease depends on the serologic methodology chosen [35-37].

Our PCR results are in disagreement with those reported by Blasi *et al.* which showed that *C. pneumoniae* DNA detection is associated with higher rates of exacerbation and airway

microbial colonisation in patients with COPD [38]. Of the 42 patients enrolled, those whose respiratory samples were *C. pneumoniae* DNA PCR positive (38%) had a significantly greater number of pathogens on sputum culture than PCR negative patients. Blasi *et al.* also found that *C. pneumoniae* positive patients (in stable COPD) had a greater tendency towards frequent exacerbation, although this difference in exacerbation frequency between the two groups was small (0.6 exacerbations per year). In a smaller, but similar study, Seemungul *et al.* found no relationship between *C. pneumoniae* DNA detection in the airway at exacerbation and exacerbation frequency [39]. The reported discrepancy in PCR positivity on respiratory samples between our study and that of Blasi *et al.* may be due to a number of reasons, including differences in PCR techniques used (real-time instead of conventional, differences in DNA polymerases, decontaminate with dUTP-uracil-DNA glycosylase, the use of specific probes, inclusion of sufficient controls, and the use of molecular grade water [35, 36]), and differences in study design and study population. In our view, real-time PCR is the current standard in the clinical microbiology laboratory, and the question of cross-contamination occurs whenever a nested PCR is performed. In this sense, the results of studies that use nested PCR to determine the prevalence of *C. pneumoniae* will always be questionable [40].

We only included patients with less severe COPD (GOLD classification of severity stage I and II) and therefore the prevalence of atypical pathogens might be different in patients with more severe disease. However, we don't think this explains the differences found in our study as compared to other serology based studies. In our view, the serological evidence of *C. pneumoniae*, *Legionella* spp. *and M. pneumoniae* playing a role as a pathogen or co-pathogen in AECOPD simply reflects the principal methodological problems of diagnosing such infections. The use of a less specific diagnostic method for the detection of a pathogen, will, from a statistical point of view, increase the likelihood of false-positive

reactions. Concern for serology specificity is even higher when a large percentage of other co-infecions are also serologically identified [7-10].

Although it is difficult to draw general conclusions or even indications for standard care on the basis of the results of this study, our data provides indirect evidence against the clinical practice of prescribing antibiotics to patients with AECOPD. From the results of our study we can conclude that in patients with less severe COPD antibiotics directed at atypical pathogens are not necessary. However, prospective, controlled trials are needed to really address the question of the effectiveness of antibiotic therapy directed against atypical respiratory pathogens and AECOPD.

In summary, in search for an association between presence of atypical pathogens in patients with stable COPD and in those with AECOPD using real-time PCR we found no indication for a role of *Legionella* spp., *C. pneumoniae* or *M. pneumoniae* in stable moderately severe COPD and in its exacerbations. This study indicates that nonstandardized serology might introduce a false association between atypical pathogens and AECOPD.

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