Eur Respir J 2007; 30: 240–244
DOI: 10.1183/09031936.00012707

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

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

The presence of these atypical pathogens in sputum samples was investigated in patients with stable COPD and with acute exacerbations of COPD using real-time PCR. The present 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 samples obtained during stable disease (stable-state sputa) and 126 samples obtained during acute exacerbations of COPD (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.

The possible relationship between the presence of atypical pathogens and the aetiology of acute exacerbations in chronic obstructive pulmonary disease was investigated in patients with stable disease and in those with acute exacerbations using real-time PCR. No indication was found of a role for Legionella spp., *Chlamydia pneumoniae* or *Mycoplasma pneumoniae* in stable, moderately severe chronic obstructive pulmonary disease and in its exacerbations.

KEYWORDS: Atypical pathogens, *Chlamydia pneumoniae*, chronic obstructive pulmonary disease, exacerbation, Legionella, real-time PCR

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality in adults. According to the Global Burden of Disease Study [1], COPD is the fifth most common disease and the fourth leading cause of death in the world. The chronic course of this disease is frequently accompanied by acute exacerbations, characterised 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]. Morbidity and mortality in COPD patients are, for the most part, related to acute exacerbations of COPD (AECOPD), which occur one to three times a year on average.

The aetiology of AECOPD is heterogeneous and still under discussion. For many years, there has been controversy regarding 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 pneumoniae, Chlamydia pneumoniae* and *Legionella pneumophila*. The role of these bacteria in AECOPD remains unclear. Serological studies suggest that these atypical pathogens may play an important role in

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Received:
February 03 2007
Accepted after revision:
April 12 2007

STATEMENT OF INTEREST
None declared.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

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 PCR, have become useful tools for the aetiological diagnosis of lower respiratory tract infections [18]. PCR can detect minute amounts of nucleic acids from potentially all PPMO; it does not depend on the viability of the target microbe; is probably less affected by previous antimicrobial therapy than culture-based methods are; 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].

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

PATIENTS AND METHODS

Patients

Patients were recruited in the period May 1999–March 2000 from the outpatient pulmonary clinic of the Medisch Spectrum Twente, a 1,150-bed teaching hospital in Enschede, the Netherlands, as previously described elsewhere [5, 20]. Inclusion criteria were as follows. 1) A clinical diagnosis of stable COPD, as defined by the American Thoracic Society criteria. 2) No history of asthma. 3) No exacerbation in the month prior to enrolment. 4) Being a current or former smoker. 5) Age 40–75 yrs. 6) A baseline pre-bronchodilator forced expiratory volume in one second (FEV_1) of 25–80% predicted. 7) A pre-bronchodilator ratio of FEV_1 to inspiratory vital capacity value of $\leqslant$60%. 8) A reversibility value of FEV_1 $\leqslant$12% pred post-inhalation of 80 μg of ipratropium bromide *via* a metered-dose inhaler with Aerochamber® (Boehringer Ingelheim, Alkmaar, the Netherlands). 9) A total lung capacity (TLC) >TLC pred-1.64 × SD. 10) No maintenance treatment with oral steroids or antibiotics. 11) No medical condition with a low survival rate or serious psychiatric morbidity (*e.g.* cardiac insufficiency or alcoholism). 12) Absence of any other active lung disease (*e.g.* sarcoidosis). The hospital's medical ethical committee approved the present study. All patients provided written informed consent.

Study protocol

The present study was part of a randomised, double-blind, single-centre study, investigating the role of inhaled corticosteroids in COPD [20]. From the 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 due to AECOPD. Clinically, exacerbations were defined as worsening of respiratory symptoms that resulted in the patient contacting the study office and receiving treatment by the study physician.

Sputum samples

Sputa were collected at scheduled visits to the outpatient department and at visits due to cases of exacerbation. Spontaneously expectorated sputum was collected in sterile vials and processed in the laboratory within 4 h of collection. Total sputum samples were homogenised 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·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 a 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 variable domain 2 and 4 of the outer membrane protein A 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 macrophage infectivity potentiator (*mip*) gene. The primers of the first Legionella spp. PCR-probe assay are based on the primers described by LINDSAY *et al.* [22], and detected in real time using a TaqMan probe Leg5S (Applied Biosystems, Nieuwerkerk a/d Ijssel, the Netherlands) [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, primers 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 base pairs amplicon. Real-time detection was performed with a fluorescent (*i.e.* labelled with 6-carboxy-fluorescein (FAM)) Taqman probe (5′-TCCCCC GTT GAA AAA GTG AGT GGG T-3′-FAM). Real-time PCR for all assays was performed on a ABi Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Results were expressed as threshold cycle (Ct) values, 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 the fluorescent signal was observed after 50 cycles, the sample was assumed to be negative.

RESULTS

104 patients (median (range) age 63 (45–75) yrs; 86 males) provided a total of 248 sputum samples. 122 samples were obtained during stable disease (stable-state sputa) and 126 samples were obtained during AECOPD (exacerbation sputa). 76 patients provided both stable-state and AECOPD samples, 18 patients provided stable-state samples only and 10 patients provided AECOPD samples only. Since a substantial proportion of patients were unable to spontaneously expectorate an adequate sputum sample, and also because only microscopically representative sputum samples were used, 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 nonpneumophila 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 nonpneumophila 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 to a level of growth of $>10^5$ colony-forming units·mL^{-1}.

DISCUSSION

The possible relationship between the presence of atypical pathogens and the aetiology of AECOPD in patients with stable COPD and in those with AECOPD was investigated using real-time PCR. No indication was found of a role for 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. Regarding microbial patterns and their possible involvement in the aetiology of AECOPD, it is a common view that *H. influenzae*, *S. pneumoniae* and *M. 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 sero-epidemiological studies is the correlation of serology with infection, as defined either by isolation of the organism in culture or by PCR. The lack of standardisation for the performance of serological test has made the interpretation of the published data from different laboratories about COPD patients difficult and, in the view of the present authors, debatable.

M. pneumoniae is a common cause of community-acquired pneumonia [2]. LIEBERMAN *et al.* [7] found *M. pneumoniae* to be the cause of AECOPD in 34 (14%) out of 240 hospitalised patients, a higher value than those reported in other studies about the microbiological aetiology of AECOPD. In 24 (71%) of these 34 patients, 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; in fact, the trend was actually in the opposite direction, with a mean length of hospitalisation for AECOPD that was 1.1 days less (5.3 *versus* 4.2 days). In three previous studies, *M. pneumoniae* was identified as a possible aetiological factor in 0.5–2.4% of AECOPD [26–28]. In a more recent study, SOLER *et al.* [29] did not find a single case of infection due to *M. pneumoniae* in a group of patients with severe COPD. *M. pneumoniae* is a fastidious organism, its culture is time-consuming and it lacks sensitivity. Consequently, the laboratory diagnosis of *M. pneumoniae* infection has largely relied on commercially available serological tests, most often enzyme immunoassays, microparticle agglutination assays and the complement fixation test. 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.* [9], 17% of hospital admissions due to AECOPD showed serological evidence of acute infection with Legionella spp.. The present findings are in concordance with those of SOLER *et al.* [29], who observed no serological evidence for infection with *L. pneumophila* in a group of patients with severe COPD. An exhaustive review of AECOPD studies 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 highly diagnostic, the sensitivity and specificity of the seroconversion to other species and serogroups have not been rigorously confirmed [19, 31]. Legionella colonisation 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]. Owing to 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 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 micro-immunofluorescence (MIF). The difficulties in *C. pneumoniae* serological testing are well known. Reports from different laboratories are highly variable, and adequate evaluations compared with a gold standard are lacking, which has led for calls for more standardised approaches in diagnostic testing [32]. Background rates of seropositivity by MIF can also be very high in some adult populations, sometimes >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 (Ig)G and IgA antibodies in their bloodstream than healthy controls [34]. Although initial reports were positive, more recent reports, 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 serological methodology chosen [35–37].

The present PCR results are in disagreement with those reported by BLASI *et al.* [38], which showed that *C. pneumoniae* DNA detection is associated with higher rates of exacerbation and airway microbial colonisation in patients with COPD. Of the 42 patients enrolled, individuals 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.* [38] 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·yr^{-1}). In a smaller, but similar, study SEEMUNGAL *et al.* [39] found no relationship between *C. pneumoniae* DNA detection in the airway at exacerbation and exacerbation frequency. The reported discrepancy in PCR positivity on respiratory samples between the present study and that of BLASI *et al.* [38] may be due to a number of reasons, including differences in PCR techniques used (real time instead of conventional, differences in DNA polymerases, decontamination with 2′-deoxyuridine-5′-triphosphate-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 the current authors' 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].

Only patients with less severe COPD (Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification of severity stage I and II) were included and, therefore, the prevalence of atypical pathogens might be different in patients with more severe disease. However, this does not explain the differences found in the present study as compared with other serology-based studies. The current authors believe that 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. From a statistical point of view, the use of a less specific diagnostic method for the detection of a pathogen will increase the likelihood of false-positive reactions. Concern for serology specificity is even higher when a large percentage of other co-infections 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 the present study, the current data provide indirect evidence against the clinical practice of prescribing antibiotics to patients with AECOPD. From the results of the present study it can be concluded 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 using real-time PCR to search for an association between the presence of atypical pathogens in patients with stable chronic obstructive pulmonary disease and in those patients with acute exacerbations of the disease, no indication was found of a role for Legionella spp., *Chlamydia pneumoniae* or *Mycoplasma pneumoniae* in stable, moderately severe chronic obstructive pulmonary disease and in its exacerbations. The present study indicates that nonstandardised serology might introduce a false association between atypical pathogens and acute exacerbations of chronic obstructive pulmonary disease.

ACKNOWLEDGEMENTS

The present authors would like to thank C. de Jong and I. Aarts (Laboratory of Medical Microbiology and Immunology, St Elisabeth Hospital, Tilburg, the Netherlands) for technical assistance.

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