Role of *Mycoplasma pneumoniae* infection in acute exacerbations of chronic obstructive pulmonary disease

Mandira Varma-Basil,¹ Shailendra K. D. Dwivedi,¹ Krishna Kumar,¹ Rakesh Pathak,¹ Ritika Rastogi,¹ S. S. Thukral,¹ Malini Shariff,¹ V. K. Vijayan,² Sunil K. Chhabra² and Rama Chaudhary³

Correspondence

Mandira Varma-Basil
mandirav@rediffmail.com

¹Department of Microbiology, V. P. Chest Institute, University of Delhi, Delhi, India

²Department of Respiratory Medicine, V. P. Chest Institute, University of Delhi, Delhi, India

³Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India

Eighty per cent of the cases of acute exacerbation of chronic obstructive pulmonary disease (AECOPD) have an infective aetiology, atypical bacteria including *Mycoplasma pneumoniae* accounting for 5–10 % of these. However, the importance of association of *M. pneumoniae* with episodes of AECOPD still remains doubtful. The present study was therefore undertaken to delineate the extent of involvement of *M. pneumoniae* in patients with AECOPD at a referral hospital in Delhi, India. Sputum samples and throat swabs from a total of 100 AECOPD patients attending the Clinical Research Center of Vallabhbhai Patel Chest Institute, Delhi, were collected during a 2-year period (January 2004–June 2006). The samples were investigated for the presence of aerobic bacterial pathogens and *M. pneumoniae*. Diagnosis of infection with *M. pneumoniae* was based on culture, serology, direct detection of *M. pneumoniae* specific antigen and PCR. Bacterial aetiology could be established in 16 of the 100 samples studied. *Pseudomonas* spp. were recovered from eight cases, *Streptococcus pneumoniae* from four and *Klebsiella* spp. from two cases. *Acinetobacter* sp. and *Moraxella catarrhalis* were isolated from one case each. Serological evidence of *M. pneumoniae* infection and/or detection of *M. pneumoniae* specific antigen were seen in 16 % of the cases. One case with definite evidence of *M. pneumoniae* infection also had coinfection with *Pseudomonas* spp. However, no direct evidence of *M. pneumoniae* infection was found in our study population as defined by culture isolation or PCR. In conclusion, although the serological prevalence of *M. pneumoniae* infection in our study population was significantly higher than in the control group, there was no direct evidence of it playing a role in AECOPD.

Received 12 May 2008

Accepted 27 November 2008

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is defined by the presence of irreversible or partially reversible airway obstruction in patients with chronic bronchitis or emphysema (Gross, 2001). The prolonged course of COPD is characterized by recurrent episodes of acute exacerbations, defined by a subjective increase from baseline of one or more symptoms including dyspnoea, cough, sputum production, sputum purulence and sputum tenacity (ATS, 1995). While several factors cause exacerbations of COPD (Sethi, 2000), the pathophysiology is infectious in about 80 % of cases. There are several reports in which *Mycoplasma pneumoniae* has been identified as an aetio-

logical agent in 0.5–2.4 % of acute exacerbations of chronic bronchitis and COPD (Gump *et al.*, 1976; Buscho *et al.*, 1978; Smith *et al.*, 1980; Allegra *et al.*, 1994; Lieberman *et al.*, 2001, 2003). However, some other workers did not find any evidence of *M. pneumoniae* infection in acute exacerbations of COPD (AECOPD) (Goh *et al.*, 1999; Diederer *et al.*, 2007). Thus the role of *M. pneumoniae* in AECOPD remains unclear and controversial. It is important to delineate the extent of involvement of *M. pneumoniae* in this disease syndrome in order to be able to plan empirical therapeutic strategies. To the best of our knowledge, no studies have been undertaken on the role of *M. pneumoniae* in AECOPD in Indian patients. The present study represents such an attempt, in patients attending the Clinical Research Center at Vallabhbhai Patel Chest Institute, Delhi, India.

Abbreviations: AECOPD, acute exacerbations of chronic obstructive pulmonary disease; GPA, Gelatin Particle Agglutination.

METHODS

Patients. One hundred adult AECOPD patients attending the Clinical Research Center at Vallabhbhai Patel Chest Institute, Delhi, India, between January 2004 and June 2006 were selected for the study. The inclusion criteria for the study were: a history of COPD, increased cough, dyspnoea and production of purulent sputum (ATS, 1995). The exclusion criteria were: treatment with any antibiotic for 24 h or longer within 72 h of the baseline visit, evidence of bronchiectasis and/or pneumonia, and malignancy or severe immunosuppression (Mogulkoc *et al.*, 1999). All the patients were asked to report back for follow-up 2–3 weeks after the baseline visit. However, only 57 patients reported for follow up. Convalescent sera samples were collected from all of these 57 patients. In addition, 35 healthy volunteers were included in the study as the control group. Informed consent was taken from each patient and healthy volunteer prior to inclusion in the study, following clearance from the Institutional Ethical Committee.

Specimens. Throat swabs were collected from patients, including the follow-up cases, kept in PPLO broth (Difco) and transported to the laboratory. Morning expectorated sputum samples were also collected from the patients in sterile containers. Acute and convalescent phase sera were separated from the blood samples collected from the patients and stored at -20°C for further analysis.

A Gram stain was performed to evaluate the quality of expectorated sputum. Only specimens with fewer than 10 squamous epithelial cells and 25 or more polymorphonuclear leukocytes per low-power field were processed further. All the samples were subcultured on supplemented PPLO agar (Difco) and PPLO broth, as previously described (Marmion, 1989). The samples were also subcultured on 5% sheep blood agar and MacConkey agar. Blood agar and MacConkey agar plates were incubated for 24 h at 37°C . PPLO agar plates were incubated for 2–3 weeks. Pure culture or predominant growth of a bacterial isolate on blood agar or MacConkey agar was considered significant and the bacterial isolate was identified as per standard procedures (Collee & Miles, 1989).

Serological tests. Specific IgM, IgG and IgA antibodies to *M. pneumoniae* were determined in 100 acute phase sera, 57 convalescent phase sera as well as in 35 sera collected from healthy controls, using commercial ELISAs as per the manufacturer's instructions (SERION ELISA classic). Values of $>30\text{ IU ml}^{-1}$ for IgG, $>17\text{ IU ml}^{-1}$ for IgM and $>14\text{ IU ml}^{-1}$ for IgA were considered positive according to the manufacturer's recommendations. Samples giving IgG titres of $20\text{--}30\text{ IU ml}^{-1}$, IgM titres of $13\text{--}17\text{ IU ml}^{-1}$ and IgA titres of $10\text{--}14\text{ IU ml}^{-1}$ were considered borderline positive as recommended by the manufacturer. All the samples were also screened for the presence of anti-*M. pneumoniae* antibodies by employing the Microtitre Gelatin Particle Agglutination (GPA) assay (Fujirebio).

PCR. PPLO broth (200 μl), inoculated with the throat swabs/sputum samples obtained from patients, was centrifuged for 30 min at 12 000 g. The pellet was resuspended in PBS (500 μl) and washed twice. The final pellet was resuspended in 20 μl distilled water, boiled for 15 min and centrifuged for 30 s at 10 000 g. The supernatant was used for PCR. An assay based on the P1 adhesin gene, using primers MpnA (5'-CCG CGA AGA GCA ATG AAA AAC TCC-3') and MpnB (5'-TCG AGG CGG ATC ATT TGG GGA GGT-3'), was used to obtain a 375 bp amplicon (Ramirez *et al.*, 1996).

Samples were amplified for 35 cycles. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 10 min. The amplification products were examined on agarose gels. A 100 bp molecular mass standard (Fermentas Life Sciences) was included in

each gel. Strain FH (ATCC 15531) was used as positive control and sterile distilled water as negative control for the PCR.

Antigen assay. Antigen assay was carried out, using the SERION ELISA antigen kit for *M. pneumoniae* (Serion Virion), on 75 throat swabs taken from AECOPD patients at random, which also included all patients with serological evidence of *M. pneumoniae* infection. The assay was also carried out on 35 throat swabs from healthy volunteers. The throat swabs were emulsified and stored in PPLO broth. The specimens were diluted 1:1 with the specimen buffer provided in the ELISA kit and incubated at 37°C for 1 h. The mixture was then centrifuged for 15 min and 100 μl of the supernatant was used for ELISA. The microtitre plate was kept at 37°C for 1 h after addition of the supernatant. After washing, the conjugate consisting of anti-*M. pneumoniae* antibodies was added to the sample wells. The plate was incubated at 37°C again for 30 min. 3,3',5,5'-Tetramethylbenzidine substrate solution was added to the plate and the plate was reincubated at room temperature for 20 min. The reaction was stopped by adding the stopping solution and the A_{450} was read.

Statistical analysis. Statistical comparisons were performed using the chi-square test or Fischer's exact test. The level of significance was defined as $P \leq 0.05$.

RESULTS AND DISCUSSION

Despite some studies associating *M. pneumoniae* with acute exacerbation of bronchial asthma and COPD, its role has not been unequivocally proven (Gump *et al.*, 1976; Buscho *et al.*, 1978; Smith *et al.*, 1980; Allegra *et al.*, 1994; Goh *et al.*, 1999; Lieberman *et al.*, 2001; Diederer *et al.*, 2007). The present study was an attempt to delineate the role of *M. pneumoniae* in AECOPD in patients in India.

One hundred sputum samples and 100 throat swabs were taken from the same number of patients with AECOPD. Ninety-eight per cent of the patients were adult males. Fifty-six per cent of the patients were from the age group 60–79 years. Most of the patients presented with cough (91%) and expectoration (90%). None of the patients had any other underlying disorder. However, 10 patients had been treated for tuberculosis in the past.

Bacterial aetiology could be established in 16 of the 100 samples studied. None of the sputum samples were culture-positive for *M. pneumoniae*. *Pseudomonas* spp. were recovered from eight cases, *Streptococcus pneumoniae* from four, *Klebsiella* spp. from two, while *Acinetobacter* sp. and *Moraxella catarrhalis* were isolated from one case each. In three of these cases, one each with evidence of *Klebsiella* spp., *S. pneumoniae* and *Pseudomonas* spp. infection, the *M. pneumoniae* IgG antibody titre was positive. One patient from whom *Pseudomonas* sp. was isolated had a borderline IgA level (12 IU ml^{-1}) in addition to an IgG titre of 150 IU ml^{-1} , indicating the possibility of a mixed infection. Co-infection of *M. pneumoniae* with bacterial pathogens or viruses has also been reported in earlier studies (Lieberman *et al.*, 2002).

ELISA assays for *M. pneumoniae* specific IgG, IgM and IgA antibodies were performed on all the serum samples. The IgG antibody titres were positive for 40/100 acute phase

serum samples (Table 1). The IgG titres in patients (mean 23.65 ± 2.684) were significantly higher than in normal healthy control subjects (mean 13.99 ± 1.996 ; $P < 0.05$) (Fig. 1a). Convalescent phase sera were collected from 57/100 patients studied, of which 35 samples were obtained from patients with raised IgG titres during the first visit. There was no change in the IgG titres in 52 patients; however, 5 patients with a raised baseline IgG titre showed a fourfold rise (Table 2). IgM antibody titres for all of these 5 patients were, however, negative ($< 10 \text{ IU ml}^{-1}$).

Five additional serum samples (Table 1) were positive in the IgM ELISA assay (mean 6.620 ± 0.5568). Two of these five patients were also positive for *M. pneumoniae* specific antigen, but were culture- and PCR-negative. One of the control samples had positive IgM titres (mean 6.057 ± 0.3888 ; $P > 0.05$) (Fig. 1b). The number of IgM-positive samples in our study was low since our study population consisted of adult patients ≥ 40 years of age. It has been observed that older patients having experienced multiple reinfections usually have only low or undetectable IgM concentrations (Barker *et al.*, 1990).

Raised IgA antibodies (Table 1) were seen in significantly higher numbers of patients (mean 9.170 ± 1.008) than controls (mean 4.057 ± 0.2167 ; $P < 0.05$) (Fig. 1c). Four patients with high IgA titres also had positive IgG titres, indicative of a primary *M. pneumoniae* infection.

Though none of the control subjects was positive for the GPA assay, 18 serum samples collected from AECOPD patients were positive for anti-*M. pneumoniae* antibodies by this assay. Three of these samples had a titre of $\geq 1:320$ (Table 2). In addition, all three had raised IgG titres. Moreover, in one of these three patients the convalescent serum showed a fourfold rise in the titres of IgG antibodies, indicating active *M. pneumoniae* infection. Although the use of paired serum samples collected at an interval of at least 2 weeks is ideal for the demonstration of a rising titre, which in turn indicates active infection, a few workers have also used single serum samples and considered a GPA titre of $\geq 1:320$ (Hirai *et al.*, 1991) significant.

PCR for the 375 bp fragment of the P1 adhesin gene was negative in all the 100 clinical samples studied. The possibility of PCR inhibitors in the sample interfering with the PCR assay has been indicated (Waites & Talkington, 2004). Since we had not included an internal 'inhibitors' control in our PCR assays, it would not be possible for us to assess whether PCR inhibitors had an impact on our results.

The *M. pneumoniae* specific antigen was detected in 3 of 75 throat swabs tested from AECOPD patients, while none of the samples from healthy control subjects were positive. Of the antigen-positive samples, one sample was also positive for IgG (85 U ml^{-1}). Another sample was positive for both IgM (25 U ml^{-1}) and IgG (32 U ml^{-1}) antibodies. The third sample was positive for IgM (26 U ml^{-1}) and IgG (42 U ml^{-1}) and borderline positive for IgA antibodies. However, the antigen-positive samples in our study were negative by PCR as well as culture. It is possible to detect non-specific cross-reactive antigens of other mycoplasmas in the respiratory tract (Waites & Talkington, 2004). In addition, as mentioned earlier, the possibility of PCR inhibitors interfering with the PCR assay also has to be considered (Waites & Talkington, 2004).

The criteria used to identify the possibility of *M. pneumoniae* infection in the study population are described in Table 2. On the basis of serology and antigen assay, 16% of our patients could be considered showing evidence of *M. pneumoniae* infection. This corroborates the findings of earlier studies which reported a higher seroprevalence of *M. pneumoniae* in AECOPD patients than in the healthy controls (Mogulkoc *et al.*, 1999; Park *et al.*, 2005). Another study, however, did not find any serological evidence of *M. pneumoniae* infection in severe exacerbations of COPD requiring ventilation (Soler *et al.*, 1998). A more recent study found no indication for a role of atypical bacteria, including *M. pneumoniae*, in either stable COPD or in its exacerbations (Diederer *et al.*, 2007). In our study, too, we did not get direct evidence by isolation and/or detection by PCR of *M. pneumoniae*. Although the seroprevalence of *M. pneumoniae* infection in our study population was

Table 1. Results of detection of *M. pneumoniae* antibodies by ELISA in the acute phase serum samples

Sample no.	Serological assay	Patient group ($n=100$)				Control group ($n=35$)				P-value
		No. negative	No. positive	No. borderline positive	Total positive	No. negative	No. positive	No. borderline positive	Total positive	
1	IgG	60	31	9	40	31	3	1	4	< 0.01
2	IgM	95	4	1	5	34	1	0	1	> 0.05
3	IgA	75	13	12	25	35	0	0	0	< 0.01
4	IgG + IgA	—	4	8*	12	—	0	0	0	< 0.05
5	IgG + IgM	—	0	1†	1	—	1	0	1	> 0.05
6	IgG + IgM + IgA	—	1	0	1	—	0	0	0	—

*Seven IgG-positive, IgA-borderline; one IgG-borderline, IgA-positive.

†IgM-positive, IgG-borderline.

Fig. 1. *M. pneumoniae* specific antibody titres in patients (■) and control (▲) subjects. The horizontal lines represent the mean of antibody titres in the two groups. (a) *M. pneumoniae* specific IgG titres. A value of $>30 \text{ IU ml}^{-1}$ was taken as positive. The difference in the means of IgG titres of patients and control samples was statistically significant ($P < 0.05$). (b) *M. pneumoniae* specific IgM titres. A value of $>17 \text{ IU ml}^{-1}$ was taken as a positive IgM titre. The difference in the means of IgM titres of patients and control samples was not found to be statistically significant ($P > 0.05$). (c) *M. pneumoniae* specific IgA titres in patients and control subjects. A value of $>14 \text{ IU ml}^{-1}$ was taken as a positive IgA titre. The difference in the means of IgA titres of patients and control samples was statistically significant ($P < 0.05$).

significantly higher than in the control group, the role, if any, played by *M. pneumoniae* in AECOPD could not be substantiated by culture isolation or detection by PCR. There is thus a need to undertake more studies in well-

Table 2. Criteria used to identify the possibility of recent *M. pneumoniae* infection

Criteria for diagnosis	Number of samples considered positive for <i>M. pneumoniae</i> infection
Definitive diagnosis	
1. Isolation of <i>M. pneumoniae</i> in culture	0
2. Nucleic acid amplification assay of P1 adhesin gene	0
3. Detection of <i>M. pneumoniae</i> antigen in respiratory secretions with raised IgM	2
4. Fourfold rise of IgG titres	5
Probable diagnosis	
1. Detection of <i>M. pneumoniae</i> antigen (with or without raised IgG/IgA)	1
2. Raised IgM titre in serum alone	2
3. Raised IgM and IgG titre in serum (without the presence of antigen)	0
4. Raised IgG and IgA titre	4*
5. IgG titres $\geq 100 \text{ U}$ (Uldum <i>et al.</i> , 1992)	2
6. Titres of antibodies in GPA assay $\geq 1:320$ (Hirai <i>et al.</i> , 1991)	3†

*Two samples also had rising titres of IgG antibodies.

†One sample also had rising titres of IgG antibody.

defined patient populations of AECOPD to establish the correlation between serological evidence of *M. pneumoniae* infection and isolation by culture and detection by PCR.

ACKNOWLEDGEMENTS

Financial support for this study was provided by the Indian Council of Medical Research. We thank Dr Mujeeb-ur-Rahman for providing help with the statistical analysis.

REFERENCES

- Allegra, L., Blasi, F., Centanni, S., Cosentini, R., Denti, F., Raccanelli, R., Tarsia, P. & Valenti, S. (1994). Acute exacerbations of asthma in adults: role of *Chlamydia pneumoniae* infection. *Eur Respir J* 7, 2165–2168.
- American Thoracic Society (1995). Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 152, S77–S121.
- Barker, C. E., Sillis, M. & Wreghitt, T. G. (1990). Evaluation of Serodia Myco II particle agglutination test for detecting *Mycoplasma pneumoniae* antibody: comparison with μ -capture ELISA and indirect immunofluorescence. *J Clin Pathol* 43, 163–165.
- Buscho, R. O., Saxtan, D., Shultz, P. S., Finch, E. & Mufson, M. A. (1978). Infections with viruses and *Mycoplasma pneumoniae* during exacerbations of chronic bronchitis. *J Infect Dis* 137, 377–383.
- Collee, J. G. & Miles, R. S. (1989). Tests for identification of bacteria. In *Mackie and McCartney's Practical Medical Microbiology*, 13th edn,

pp. 141–160. Edited by J. G. Collee, J. P. Duguid, A. G. Fraser & B. P. Marmion. Edinburgh: Churchill Livingstone.

Diederer, B. M., van der Valk, P. D., Kluytmans, J. A., Peeters, M. F. & Hendrix, R. (2007). The role of atypical respiratory pathogens in exacerbations of chronic obstructive pulmonary disease. *Eur Respir J* **30**, 240–244.

Goh, S. K., Johan, A., Cheong, T. H. & Wang, Y. T. (1999). A prospective study of infections with atypical pneumonia organisms in acute exacerbations of chronic bronchitis. *Ann Acad Med Singapore* **28**, 476–480.

Gross, N. J. (2001). The GOLD standard for chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **163**, 1047–1048.

Gump, D. W., Phillips, C. A., Forsyth, B. R., McIntosh, K., Lamborn, K. R. & Stouch, W. H. (1976). Role of infection in chronic bronchitis. *Am Rev Respir Dis* **113**, 465–474.

Hirai, Y., Shiode, J., Masayoshi, T. & Kanemasa, Y. (1991). Application of an indirect immunofluorescence test for detection of *Mycoplasma pneumoniae* in respiratory exudates. *J Clin Microbiol* **29**, 2007–2012.

Lieberman, D., Lieberman, D., Ben-Yaakov, M., Lazarovich, Z., Hoffman, S., Ohana, B., Friedman, M. G., Dvoskin, B., Leinonen, M. & Boldur, I. (2001). Infectious etiologies in acute exacerbation of COPD. *Diagn Microbiol Infect Dis* **40**, 95–102.

Lieberman, D., Lieberman, D., Ben-Yaakov, M., Shmarkov, O., Gelfer, Y., Varshavsky, R., Ohana, B., Lazarovich, Z. & Boldur, I. (2002). Serological evidence of *Mycoplasma pneumoniae* infection in acute exacerbation of COPD. *Diagn Microbiol Infect Dis* **44**, 1–6.

Lieberman, D., Lieberman, D., Printz, S., Ben-Yaakov, M., Lazarovich, Z., Ohana, B., Friedman, M. G., Dvoskin, B., Leinonen, M. & Boldur, I. (2003). Atypical pathogen infection in adults with acute exacerbation of bronchial asthma. *Am J Respir Crit Care Med* **167**, 406–410.

Marmion, B. P. (1989). Mycoplasma: Achleoplasma: Ureaplasma. In *Mackie and McCartney's Practical Medical Microbiology*, 13th edn,

pp. 745–768. Edited by J. G. Collee, J. P. Duguid, A. G. Fraser & B. P. Marmion. Edinburgh: Churchill Livingstone.

Mogulkoc, N., Karakurt, S., Isalska, B., Bayindir, U., Celikel, T., Korten, V. & Colpan, N. (1999). Acute purulent exacerbation of chronic obstructive pulmonary disease and *Chlamydia pneumoniae* infection. *Am J Respir Crit Care Med* **160**, 349–353.

Park, S. J., Lee, Y. C., Rhee, Y. K. & Lee, H. B. (2005). Seroprevalence of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in stable asthma and chronic obstructive pulmonary disease. *J Korean Med Sci* **20**, 225–228.

Ramirez, J. A., Ahkee, S., Tolentino, A., Miller, R. D. & Summersgill, J. T. (1996). Diagnosis of *Legionella pneumophila*, *Mycoplasma pneumoniae* or *Chlamydia pneumoniae* lower respiratory infection using the polymerase chain reaction on a single throat swab specimen. *Diagn Microbiol Infect Dis* **24**, 7–14.

Sethi, S. (2000). Infectious etiology of acute exacerbations of chronic bronchitis. *Chest* **117**, 380S–385S.

Smith, C. B., Golden, C. A., Kanner, R. E. & Renzetti, A. D., Jr (1980). Association of viral and *Mycoplasma pneumoniae* infections with acute respiratory illness in patients with chronic obstructive pulmonary diseases. *Am Rev Respir Dis* **121**, 225–232.

Soler, N., Torres, A., Ewig, S., Gonzalez, J., Celis, R., El-Ebiary, M., Hernandez, C. & Rodriguez-Roisin, R. (1998). Bronchial microbial patterns in severe exacerbations of chronic obstructive pulmonary disease (COPD) requiring mechanical ventilation. *Am J Respir Crit Care Med* **157**, 1498–1505.

Uldum, S. A., Jensen, J. S., Sondergard-Andersen, J. & Lind, K. (1992). Enzyme immunoassay for detection of immunoglobulin M (IgM) and IgG antibodies to *Mycoplasma pneumoniae*. *J Clin Microbiol* **30**, 1198–1204.

Waites, K. B. & Talkington, D. F. (2004). *Mycoplasma pneumoniae* and its role as a human pathogen. *Clin Microbiol Rev* **17**, 697–728.