*The Journal of Laryngology & Otology (2015)*, ***129***, *865–869*.

doi:10.1017/S0022215115001590

**MAIN ARTICLE**

# *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*: a comparative study in patients with nasal polyposis and healthy controls

D G IOANNIDIS[1], V A LACHANAS[2], Z FLOROU[1], J G BIZAKIS[2], E PETINAKI[1], C E SKOULAKIS[2]

[1]*Department of Microbiology, University of Thessaly, Larissa, and* [2]*Department of Otorhinolaryngology, University Hospital of Larissa, Greece*

## Abstract

*Introduction*: The role played by *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in the pathogenesis of chronic rhinosinusitis with nasal polyps has been the object of ongoing debate. We used real-time polymerase chain reaction to investigate the prevalence of both microorganisms in the nasal tissue samples of patients and controls.

*Methods*: We extracted DNA from nasal polyp samples obtained during functional endoscopic sinus surgery and the inferior turbinate samples of controls undergoing septoplasty. We used the highly sensitive real-time polymerase chain reaction to detect the presence of *M pneumoniae* and *C pneumoniae* DNA.

*Results*: Patients with chronic rhinosinusitis with nasal polyps consisted of 62 individuals (39 men; mean age 51 years); the control group consisted of 24 individuals (13 men; mean age 45 years). All samples from both groups were negative for *M pneumoniae* and *C pneumoniae* DNA.

*Conclusion*: We have demonstrated that the likelihood of *M pneumoniae* and *C pneumoniae* acting as an ongoing inflammatory stimulus in chronic rhinosinusitis with nasal polyps is slim.

**Key words:** Nasal Polyps; *Mycoplasma pneumoniae*; *Chlamydophila pneumoniae*; Real-Time Polymerase Chain Reaction

## Introduction

Chronic rhinosinusitis is characterised by chronic inflammation of the sinonasal mucosa that persists for at least 12 weeks despite medical therapy.[1] It is one of the most prevalent chronic diseases, affecting approximately 11 per cent of the European population.[2] Chronic rhinosinusitis is often divided into two clinically and phenotypically distinct subtypes: chronic rhinosinusitis with and without nasal polyps.[1] Unlike acute bacterial sinusitis, whose pathophysiology is well defined, it is increasingly acknowledged that chronic rhinosinusitis is not a single disease entity. Instead, it is a heterogeneous condition broadly characterised by persistent inflammation of the sinonasal mucosa.[3] The causes of such inflammation are diverse and multifactorial, relating to overlapping host and environmental triggers.[3]

*Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* are bacteria known to cause respiratory tract infections.[4] Both have been associated with community-acquired pneumonia and acute bronchitis, and with persistent asthma and chronic obstructive pulmonary disease (COPD) infections.[4–10] Taking into account the unified airway theory,[10] which considers allergic rhinitis, rhinosinusitis and COPD as manifestations of the same inflammatory process within a continuous airway, it is also possible that these bacteria may cause persistent infection of the sinus mucosa in patients with chronic rhinosinusitis with nasal polyps.

A few studies have investigated the potential role of *M pneumoniae*[11–14] and *C pneumoniae*[9,13,15–17] in chronic rhinosinusitis with nasal polyps using different techniques, although their results are controversial. In our study, we used real-time polymerase chain reaction to evaluate and compare the prevalence of *M pneumoniae* and *C pneumoniae* DNA in the nasal tissue samples of patients with chronic rhinosinusitis with nasal polyps and in those from healthy controls. To our knowledge, real-time polymerase chain reaction has not previously been used to assess the prevalence of *M pneumoniae* and *C pneumoniae* DNA in nasal tissue samples.

## Methods

We conducted a prospective study from May 2010 to May 2014, involving adult patients with chronic rhinosinusitis with nasal polyps undergoing functional endoscopic sinus surgery (FESS). We made a diagnosis of

Accepted for publication 2 February 2015 First published online 30 July 2015

chronic rhinosinusitis with nasal polyps according to the criteria set out in the European Position Paper on Rhinosinusitis and Nasal Polyps.[1] The control group consisted of healthy adult patients without chronic rhinosinusitis, but with nasal septal deviation undergoing septoplasty as outlined by the criteria.[1] We excluded from the study pediatric patients, and patients with cystic fibrosis, primary ciliary dyskinesia, allergic fungal sinusitis, inverted papilloma and human immunodeficiency virus seropositivity. We also excluded individuals in both groups who had had an upper respiratory tract infection within two weeks before surgery, and those who had taken any topical or systemic steroids within the last month prior to surgery.

In patients with chronic rhinosinusitis with nasal polyps, we obtained nasal polyp specimens during FESS; in the control group, we obtained tissue biopsies from the inferior turbinate mucosa during septoplasty. We immediately transferred the specimens to sterile, dry containers and shipped them to the laboratory. Using a surgical knife, we cut the tissues into several 2–4-mm-thick pieces (for the superficial to deep areas respectively) and then divided them into two groups: one to be used for the molecular assays and the other to be stored at −80° C.

We chose four pieces of tissue from each patient, two superficial and two deep, for DNA extraction with commercial kits (Life Technologies Ltd, Paisley, Scotland, UK), according to the manufacturer's instructions. The efficiency of DNA extraction and the possible presence of inhibitors in each sample were confirmed by the RS42 (5′-GCTCACTCAGTGTGGCAAAG-3′) and Km (5′-GGTTGGCCAATCTACTCCCAGG-3′) primers having detected the *beta 2-globin* gene.

We tested the extracted DNA specimens for the presence of *M pneumoniae* and *C pneumoniae* DNA with the Applied Biosystems 7500 fast real-time polymerase chain reaction system (Life Technologies Ltd, Paisley, UK), using a quantitative polymerase chain reaction Alert Kit (ELITech Group, Puteaux, France) for *M pneumoniae* and *C pneumoniae*, according to the manufacturer's instructions.

We entered all data into a database, including patient demographic information (age, gender, history) and data on comorbidities like allergy, asthma and COPD.

All samples were analysed at the Department of Microbiology, University of Thessaly, Greece. The University of Thessaly institutional review board approved the study. We obtained written informed consent from all patients and controls.

All procedures contributing to this work comply with the ethical standards of the relevant national and institutional guidelines on human experimentation of the University of Thessaly and with the Declaration of Helsinki (1975), as revised in 2008.

## Results

The nasal polyps group consisted of 62 individuals (39 men; mean age 51 years; range 21–74 years), while the control group consisted of 24 individuals (13 men; mean age 45 years; range 18–52 years).

DNA extraction, indicated by *beta 2-globin* gene detection, was successful in all the samples.

All specimens in both nasal polyp and control groups were negative for *M pneumoniae* and *C pneumoniae* DNA, so we conducted no further statistical analysis.

## Discussion

In 1996, Gurr *et al.*[11] were the first to investigate the potential role of *M pneumoniae* in the aetiology of nasal polyps using molecular techniques (polymerase chain reaction). In a pilot study involving 14 patients with chronic rhinosinusitis with nasal polyps, 5 patients with chronic rhinosinusitis without nasal polyps and 7 controls, they reported that *M pneumoniae* DNA was detected in 13/14 (93 per cent) of nasal polyp samples and 4/5 (80 per cent) of chronic rhinosinusitis without nasal polyp samples, but only in 1/7 (14 per cent) of the controls (inferior turbinates). They went on to suggest a *M pneumoniae* causative relationship in the aetiology of nasal polyps. Nevertheless, in a follow-up study, Bucholtz *et al.*[12] failed to detect *M pneumoniae* DNA in nasal tissue from patients and controls. The authors tested 40 patients with chronic rhinosinusitis with nasal polyps, 6 patients with chronic rhinosinusitis without nasal polyps and 9 controls (inferior turbinates) for the presence of bacterial DNA. They used *M pneumoniae*-specific DNA primers to encode the *16S ribosomal RNA* gene in 41 specimens (31 chronic rhinosinusitis with nasal polyps, 4 chronic rhinosinusitis without nasal polyps and 6 controls); in addition, they used the consensus sequence-based polymerase chain reaction with broad-spectrum primers for most eubacterial DNA encoding of the *16S ribosomal RNA* gene in 38 specimens (26 chronic rhinosinusitis with nasal polyps, 5 chronic rhinosinusitis without nasal polyps and 7 controls). They found no evidence of *M pneumoniae*-specific DNA encoding of the *16S ribosomal RNA* gene in any of the tissues. Recently, Pandak *et al.*[13] used real-time polymerase chain reaction in sinus lavage aspirates but failed to detect any *M pneumoniae* DNA in 60 chronic rhinosinusitis patients (24 chronic rhinosinusitis without nasal polyps, 36 chronic rhinosinusitis with nasal polyps). Nia *et al.*,[14] using a polymerase chain reaction-enzyme-linked immunosorbent assay, reported *M pneumoniae* DNA positivity in 19.6 per cent of biopsy specimens from chronic rhinosinusitis with nasal polyps patients (10/51 specimens) in comparison to none in 19 controls (patients undergoing surgery for nasal fractures), adding support to the initial findings of Gurr *et al.*[11] In our study, using molecular assays, we tested the presence of *M pneumoniae* and *C pneumoniae* in biopsy specimens from patients with chronic rhinosinusitis with nasal polyps and controls. Although the molecular methods we used were characterised by high sensitivity, we did not find any *M pneumoniae* positivity in either group (Table I).

https://doi.org/10.1017/S0022215115001590 Published online by Cambridge University Press

TABLE I
ENGLISH-LANGUAGE STUDIES ON THE PRESENCE OF *MYCOPLASMA PNEUMONIAE* IN CHRONIC SINUSITIS WITH AND WITHOUT NASAL POLYPS

| | *Mycoplasma pneumoniae* | | | |
|---|---|---|---|---|
| | Chronic rhinosinusitis with nasal polyps (positive/*n*) | Chronic rhinosinusitis without nasal polyps (positive/*n*) | Controls (positive/*n*) | Technique |
| Gurr *et al.*[11] | 13/14* | 4/5* | 1/7* | PCR |
| Bucholtz *et al.*[12] | 0/40* | 0/6* | 0/9* | PCR |
| Pandak *et al.*[13] | 0/36† | 0/24† | N/A | Real-time PCR |
| Nia *et al.*[14] | 10/51* | N/A | 0/19* | PCR |
| Ioannidis *et al.* (this study) | 0/62* | N/A | 0/24* | Real-time PCR |

*Tissue samples.
†Aspirates from sinus lavage.
PCR = polymerase chain reaction; N/A = not applicable

Therefore, our results suggest that *M pneumoniae* does not play a role in the aetiology of chronic sinusitis with nasal polyps.

The potential association of *C pneumoniae* with the pathogenesis of nasal polyposis was initially investigated by Lee *et al.*[15] In a pilot study, the researchers used qualitative polymerase chain reaction and demonstrated the absence of *C pneumoniae* in samples from 11 chronic rhinosinusitis patients and 6 cadaveric controls. In contrast, Apan *et al.*[9] used indirect immunofluorescence and demonstrated a statistically significant difference ($p = 0.034$) in *C pneumoniae* prevalence among patients with chronic rhinosinusitis with nasal polyps (16/30, 53.3 per cent) *vs* age- and sex-matched controls (8/30, 26.6 per cent). Subsequent studies that used qualitative polymerase chain reaction to analyse biopsy samples reported contradictory findings. While Fahrenholz *et al.*[16] failed to find evidence of *C pneumoniae* colonisation with qualitative polymerase chain reaction analysis in 27 patients with medically refractory chronic rhinosinusitis and 5 controls, Shokrollahi *et al.*[17] reported *C pneumoniae* DNA positivity in 10/51 (19.6 per cent) samples from chronic rhinosinusitis with nasal polyps patients versus 0/19 (0 per cent) in controls. The major limitation of these studies was the small number of patients included.

A study with a larger sample was performed by Pandak *et al.*[13] They used real-time polymerase chain reaction in sinus lavage aspirates, but failed to detect any *C pneumoniae* DNA in 60 chronic rhinosinusitis patients (24 chronic rhinosinusitis without nasal polyps and 36 chronic rhinosinusitis with nasal polyps). The main limitation of their study, however, was that polymerase chain reaction testing was performed on aspirates obtained during FESS and not on tissue biopsy specimens, as in the other studies. Given the intracellular life cycle of *C pneumoniae* and the documented increased presence of amplification inhibitors in aspirates[18] compared to tissue specimens, this would have influenced the study findings. Our study confirms the findings of Pandak *et al.*,[13] who argued against *C pneumoniae* involvement in the pathogenesis of chronic rhinosinusitis with nasal polyps (Table II).

TABLE II
ENGLISH-LANGUAGE STUDIES ON THE PRESENCE OF *CHLAMYDOPHILA PNEUMONIAE* IN CHRONIC RHINOSINUSITIS WITH AND WITHOUT POLYPS

| | *Chlamydophila pneumoniae* | | | | |
|---|---|---|---|---|---|
| | Chronic rhinosinusitis with nasal polyps (positive/*n*) | Chronic rhinosinusitis without nasal polyps (positive/*n*) | Chronic rhinosinusitis (positive/*n*) | Controls (positive/*n*) | Technique |
| Apan *et al.*[9] | 16/30* | N/A | N/A | 08/30* | Indirect immunofluorescence |
| Pandak *et al.*[13] | 0/36† | 0/24† | 0/60† | N/A | Real-time PCR |
| Lee *et al.*[15] | N/A | N/A | 0/11* | 0/6* | PCR |
| Fahrenholz *et al.*[16] | N/A | N/A | 0/27* | 0/5* | PCR |
| Shokrollahi *et al.*[17] | 10/51* | N/A | N/A | 0/19* | PCR |
| Ioannidis *et al.* (this study) | 0/62* | N/A | N/A | 0/24* | Real-time PCR |

*Tissue samples.
†Aspirates from sinus lavage.
PCR = polymerase chain reaction; N/A = not applicable

https://doi.org/10.1017/S0022215115001590 Published online by Cambridge University Press

Diagnosis of *M pneumoniae* and *C pneumoniae* infection is usually based on detection in the serum of specific antibodies against these microorganisms, while other conventional methods, such as culturing and antigen detection, are not used.[19] It should be noted, however, that molecular assay sensitivity has some limitations. Specifically, potential contamination during sample processing may lead to false positive results, while the presence of inhibitory factors in the samples[20,21] may produce false negative results. Moreover, specimen type is crucial for polymerase chain reaction-based diagnosis of atypical pathogens in infected patients; sputum gives superior results than nasopharyngeal specimens and throat swabs in the diagnosis of community-acquired pneumonia caused by atypical bacteria.[22]

- **Chronic rhinosinusitis is a heterogeneous condition broadly characterised by persistent inflammation of the sinonasal mucosa**
- **Its causes are diverse and multifactorial, relating to overlapping host and environmental triggers**
- **Only a few studies have investigated the potential role of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in chronic rhinosinusitis with nasal polyps**
- **We used real-time polymerase chain reaction to investigate the role of *M pneumoniae* and *C pneumoniae* in the aetiology of chronic rhinosinusitis with nasal polyps**
- **Our findings demonstrate that it is unlikely that *M pneumoniae* and *C pneumoniae* act as an ongoing inflammatory stimulus in chronic rhinosinusitis with nasal polyps**

Aspirates are more likely to be rejected in comparison to throat specimens as they may contain inadequate material or excess amplification inhibitors,[18] and thus are more likely to produce false negative results. Additionally, detection of DNA may yield different, time-dependent results during infection, as observed in pneumonia caused by atypical community-acquired pathogens; thus, the timing of sampling in each of the studies may have influenced the results.[22] *Mycoplasma pneumoniae* infection has been known to exhibit outbreaks occurring every 4–7 years and is significantly more common during the winter months.[23] Nilsson *et al*.[20] assessed the longitudinal follow-up of *M pneumoniae* infection and established that although most patients have detectable *M pneumoniae* DNA in throat secretions for seven weeks after the onset of illness, and several continue to be polymerase chain reaction-positive for several months, the *M pneumoniae* DNA load gradually declines over time, and eventually all patients became polymerase chain reaction-negative. This pattern is in contrast to a transition from active *M pneumoniae* clinical infection to a state of chronic colonisation, lowering the likelihood that *M pneumoniae* acts as the ongoing inflammatory stimulus in patients with chronic rhinosinusitis with polyps. With regard to the seasonal and widespread occurrence of *M pneumoniae* infection, it offers a possible explanation for the variability in prevalence among studies.

The potential limitations of polymerase chain reaction testing, together with differences in study populations and techniques used, could offer a logical explanation for the inter-study variability noted in the studies discussed earlier. In the present study, we took all the necessary precautions to avoid these limitations, by choosing to test a large population of biopsy specimens compared to aspirates with a quantitative-polymerase chain reaction kit employing the TaqMan® minor groove binder probe (iCycler, Bio-Rad Laboratories, Herculus, CA, USA). This technology incorporates minor groove binder molecules to increase specificity, uracil-N-glycosylase to prevent false positive results, together with a passive reference dye to calibrate the basic fluorescence and an internal control to avoid false negative results.

## Conclusion

Our findings demonstrated a complete absence of *M pneumoniae* and *C pneumoniae* DNA in biopsy specimens obtained from patients with chronic rhinosinusitis with nasal polyps and controls. These findings agree with and add further support to those arguing against atypical bacteria implication in chronic rhinosinusitis with nasal polyps. However, we cannot exclude that the inter-study variability is due to differences in study populations, nor the possibility that *M pneumoniae* and *C pneumoniae* may play a role either as potential causes of the initial development of inflammation – as in allergy and asthma – or act as modifiers of the disease process – as an ongoing inflammatory stimulus. Future research should employ a long-term prospective study design to assess the likelihood of chronic rhinosinusitis with nasal polyps prevalence in patients with documented atypical bacterial infection compared to healthy individuals.

## Acknowledgements

We thank Timoleon F Terzis and Paraskevi Tsirevelou for the assistance they provided in sample collection, and for their advice and continuous support throughout the entire project.

### References

1 Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F *et al*. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 2012;**50**:1–12
2 Hastan D, Fokkens WJ, Bachert C, Newson RB, Bislimovska J, Bockelbrink A *et al*. Chronic rhinosinusitis in Europe: an underestimated disease. A GA$^2$LEN study. *Allergy* 2011;**66**:1216–23
3 Lee S, Lane AP. Chronic rhinosinusitis as a multifactorial inflammatory disorder. *Curr Infect Dis Rep* 2011;**13**:159–68
4 Blasi F. Atypical pathogens and respiratory tract infections. *Eur Respir J* 2004;**24**:171–81
5 Ye Q, Xu XJ, Shao WX, Pan YX, Chen XJ. *Mycoplasma pneumoniae* infection in children is a risk factor for developing allergic diseases. *ScientificWorldJournal* 2014;**2014**:986527

https://doi.org/10.1017/S0022215115001590 Published online by Cambridge University Press

6 Yano T, Ichikawa Y, Komatu S, Arai S, Oizumi K. Association of *Mycoplasma pneumoniae* antigen with initial onset of bronchial asthma. *Am J Respir Crit Care Med* 1994;**149**:1348–53
7 Sutherland ER, Martin RJ. Asthma and atypical bacterial infection. *Chest* 2007;**132**:1962–6
8 Hammerschlag MR. The intracellular life of chlamydiae. *Semin Pediatr Infect Dis* 2002;**13**:239–48
9 Apan TZ, Alpay D, Alpay Y. The possible association of *Chlamydia pneumoniae* infection with nasal polyps. *Eur Arch Otorhinolaryngol* 2007;**264**:27–31
10 Meltzer EO, Szwarcberg J, Pill MW. Allergic rhinitis, asthma, and rhinosinusitis: diseases of the integrated airway. *J Manag Care Pharm* 2004;**10**:310–7
11 Gurr PA, Chakraverty A, Callanan V, Gurr SJ. The detection of *Mycoplasma pneumoniae* in nasal polyps. *Clin Otolaryngol Allied Sci* 1996;**21**:269–73
12 Bucholtz GA, Salzman SA, Bersalona FB, Boyle TR, Ejercito VS, Penno L *et al*. PCR analysis of nasal polyps, chronic sinusitis, and hypertrophied turbinates for DNA encoding bacterial 16S rRNA. *Am J Rhinol* 2002;**16**:169–73
13 Pandak N, Pajić-Penavić I, Židovec-Lepej S, Planinić A, Trošelj-Vukić B, Perić L. *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* were not identified in sinus mucosa of patients with chronic rhinosinusitis. *Eur Arch Otorhinolaryngol* 2014;**271**:1553–5.
14 Nia SJ, Farhadi M, Darestani SG, Tabatabaei A, Shamshiri AR, Noorbakhsh S. Searching the *M. Pneumonia* in cases with nasal polyp accompanied by chronic rhinosinusitis using polymerase chain reaction in tissue and serology: a case control study. *Open Access Scientific Reports* 2013;**2**:711. In: http://omicsonline.org/scientific-reports/2155-9597-SR-711.pdf [3 July 2015]
15 Lee RE, Kaza S, Plano GV, Casiano RR. The role of atypical bacteria in chronic rhinosinusitis. *Otolaryngol Head Neck Surg* 2005;**133**:407–10
16 Fahrenholz J, Stratton C, Wolf B, Duncavage J, Tang Y, Russell P *et al*. *Chlamydophila pneumoniae* and chronic sinusitis. *J Allergy Clin Immunol* 2008;**121**:S217
17 Shokrollahi MR, Farhadi M, Noorbakhsh S, Nia SJ, Ghavidel S, Shamshiri AR. Role of *C. Pneumoniae* in nasal polyp formation: PCR in tissue and serology: a cross sectional study: Tehran, Iran. *Int J Curr Microbiol App Sci* 2014;**3**:754–9
18 Reznikov M, Blackmore TK, Finlay-Jones JJ, Gordon DL. Comparison of nasopharyngeal aspirates and throat swab specimens in a polymerase chain reaction-based test for *Mycoplasma pneumoniae*. *Eur J Clin Microbiol Infect Dis* 1995;**14**:58–61
19 Kuoppa Y, Boman J, Scott L, Kumlin U, Eriksson I, Allard A. Quantitative detection of respiratory *Chlamydia pneumoniae* infection by real-time PCR. *J Clin Microbiol* 2002;**40**:2273–4
20 Nilsson AC, Björkman P, Persson K. Polymerase chain reaction is superior to serology for the diagnosis of acute *Mycoplasma pneumoniae* infection and reveals a high rate of persistent infection. *BMC Microbiol* 2008;**8**:93
21 Gibb AP, Wong S. Inhibition of PCR by agar from bacteriological transport media. *J Clin Microbiol* 1998;**36**:275–6
22 Cho MC, Kim H, An D, Lee M, Noh SA, Kim MN *et al*. Comparison of sputum and nasopharyngeal swab specimens for molecular diagnosis of *Mycoplasma pneumoniae, Chlamydophila pneumoniae*, and *Legionella pneumophila*. *Ann Lab Med* 2012;**32**:133–8
23 Sidal M, Kilic A, Unuvar E, Oguz F, Onel M, Agacfidan A *et al*. Frequency of *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* infections in children. *J Trop Pediatr* 2007;**53**:225–31

Address for correspondence:
Dr Efthymia Petinaki,
Department of Microbiology,
University of Thessaly Viopolis,
PC 41110 Larissa, Greece

Fax: +30 241 3 501570
E-mail: petinaki@med.uth.gr

Dr E. Petinaki takes responsibility for the integrity of the content of the paper
Competing interests: None declared