Chlamydophila pneumoniae and Mycoplasma pneumoniae in respiratory specimens of children with chronic lung diseases

N Teig, A Anders, C Schmidt, C Rieger, S Gatemann

Background: Persistent infection with Mycoplasma pneumoniae and Chlamydophila pneumoniae has been implicated in the progression or induction of asthma and chronic obstructive pulmonary disease. Evidence for this hypothesis has been obtained in adults either by serological methods or by direct pathogen detection using invasive procedures.

Methods: We investigated nasal brush specimens and induced sputum from 38 children with stable chronic lung disease (asthma, n = 26; chronic bronchitis n = 12) and from 42 healthy controls for the presence of M pneumoniae or C pneumoniae DNA by polymerase chain reaction (PCR) using nested primers.

Results: None of the controls but 23.6% and 10.5% of the children with lung disease had positive PCR for C pneumoniae (p = 0.001) and M pneumoniae (p = 0.044) respectively. Significantly more children with non-atopic asthma than with atopic asthma were positive for C pneumoniae or M pneumoniae (4/8 v 1/18; p = 0.018). There were no unwanted side effects from sputum induction. No correlation was found between detection of Chlamydophila and severity of lung disease. Colonisation with both organisms had occurred before adulthood in a significant proportion of children with stable chronic lung diseases.

Conclusion: Combining nasal brush specimens with induced sputum may be a useful non-invasive method for studying the role of C pneumoniae and M pneumoniae infection in children with different chronic lung diseases.

Abbreviations: CP, Chlamydophila pneumoniae; FEV1, forced expiratory volume in 1 second; MP, Mycoplasma pneumoniae; PCR, polymerase chain reaction
hospital. They were classified as having asthma if they had had a physician’s diagnosis of asthma and recurrent (at least two) episodes of wheezing during the previous 12 months. Chronic purulent bronchitis was defined as daily sputum production for more than 2 years and at least two positive sputum cultures for conventional bacteria in this period. Atopy was defined as at least one positive specific IgE (CAP or RAST class >1) or skin prick test (weal >2 mm) to seven common inhalant allergens during the preceding 3 years. Control children were considered suitable for inclusion in the study if they had no history of recurrent wheezing or chronic sputum production, and normal lung function and auscultation of the lungs on the day of the study. They suffered from a range of non-pulmonary conditions including insulin dependent diabetes mellitus, mental and/or behavioural problems, epilepsy, resolving gastroenteritis, burn wounds in the stage of scarring, and benign haematological conditions.

Controls and patients were excluded from the study if they had received one or more courses of antibiotics during the previous 3 months with activity against Chlamydia pneumoniae or Mycoplasma (such as macrolides, tetracyclines or quinolones) or had evidence of an acute exacerbation of their underlying lung disease during this time period. Patients with immunodeficiencies were also excluded.

The study was performed for patients and controls from June to October 1999 and from April to September 2000. All children underwent lung function testing using forced expiratory flow-volume curves (Bodyscreen, Jaeger/Viasys, Germany) and results were expressed as percentages of predicted for age and length of the children.

The study was approved by the institutional review board of the medical faculty of Ruhr-University Bochum and all study procedures were performed after informed consent of the parents and the child had been obtained.

**Sputum induction and processing**

Sputum was induced using a nebuliser (Pari Boy, Starnberg, Germany) with increasing concentrations of saline (starting at 0.9% and increasing every 5 minutes up to a concentration of 5.8%). Every 5 minutes a flow-volume curve was obtained. A fall in forced expiratory volume in 1 second (FEV₁) of more than 20% from baseline was considered to be a criterion to stop the induction procedure. Children were asked to perform coughs every 2–3 minutes and the sputum was collected in a petri dish. Macroscopically visible sputum was separated from saliva and processed according to published protocols. Part of the sputum sample was weighed and mixed for 30 seconds with four times the volume of 0.1% dithiothreitol (DTT) by gentle aspiration in and out of a Pasteur pipette and rocked for 15 minutes on a bench rocker. The sample was diluted with an equal volume of Hank’s balanced salt solution (HBSS) and the resulting suspension was filtered through a 48 μm gauze. The filtered suspension was centrifuged at 30g for 10 minutes. The cell pellet was resuspended in HBSS and squamous cell contamination was determined after staining. Patients with macroscopically insufficient sputum or with differential counts containing more than 50% squamous cells were excluded from further analysis.

The rest of the unprocessed sputum was frozen at −70°C for microbiological analysis.

**Nasal specimens**

Nasal specimens were obtained by brush biopsy using a 2 mm cytology brush (BC-15 AE, Olympus) which was moistened with sterile normal saline before being introduced into the nose. The lateral inferior turbinate was brushed three times at a length of 4–5 cm. The material was suspended in normal saline and stored at −70°C until analysis. If no macroscopically visible material was recovered, the procedure was repeated. Previous studies in our laboratory have shown that this method yields sufficient cells (10⁵–10⁶ viable cells/ml) in >96% of specimens obtained from healthy school children.

**Detection of Mycoplasma pneumoniae and Chlamydia pneumoniae DNA by nested PCR**

The laboratory investigators were blind to the patients’ data.

**Mycoplasma pneumoniae**

A total of 500 μl nasopharyngeal brush specimen or induced sputum submitted from the Children’s Hospital was pretreated with 0.5% acetyl cysteine and centrifuged for 30 minutes at 15 000g at 4°C. DNA was extracted from the pellet with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The MP specific primer pair MP-1 5’-GAA GCT ATT ACC ATC TCT TTA TTT CG-3’ and MP-2 5’-ATT ACC ATC TCT TTA TTT CG-3’ (MWG-Biotech, Ebersburg, Germany) described by Bernet,25 coding for a fragment of the ATPase operon gene, was used for the first PCR. The second PCR was carried out by submitting 5 μl of 1:10 diluted PCR product from the first PCR to an amplification with the following primer pair: MUH-1 5’-TGA CTC GAA GGA TGT TAA GC-3’ and MUH-2 5’- ATT ACC ATC TCT TTA TTT CG-3’.26 Amplification reactions were performed in a volume of 50 μl containing 10 × PCR buffer with 1.5 mM MgCl₂ (Amersham Biosciences, Freiburg, Germany), 0.2 mM each of deoxynucleoside triphosphate (Amersham Biosciences), 1 μM of each primer, and 1.25 U Taq DNA polymerase (Amersham Biosciences) and 5 μl of DNA template in a Perkin Elmer GeneAmp 2400 thermocycler.

Following an initial denaturation step at 95°C for 7 minutes, the 40 cycle amplification programme consisted of denaturation at 95°C for 20 seconds, annealing at 52°C for 2 minutes for the first round and 50°C for the second round, and elongation at 72°C for 1 minute. The nested PCR generated a 104 bp product which was analysed on a 2% agarose gel containing ethidium bromide. All positive samples were reconfirmed. The sensitivity of the nested PCR product on gel electrophoresis was 6 fg M pneumoniae FH ATCC 15531 cDNA.

![Figure 1](https://www.thoraxjnl.com)
Chlamydia pneumoniae

CP PCR was performed in the same way but using different primers and a different cycling protocol. We used the outer primer pair CPHL-1 5'-GTT CAT GAA GCC CTA CT-3' and CPHR-1 5'-TGC ATA ACC TAC GTG TT-3' which is specific for CP and the inner primer pair CPHn-1 5'-AGT TGA GCA TAT TCG TGA GG-3' and CPHn-2 5'-TTT ATT ATT TCC GTG TCG TCC AG-3'. These primers amplify a sequence of unknown function of CP and do not amplify DNA from normal flora or any other respiratory pathogen.

DNA amplification consisted of 32 cycles of 95°C for 1:30 min, 55°C for 1:45 min and 72°C for 1:45 min, after a pre-incubation at 95°C for 2 minutes. The resulting products from the first and second PCR had sizes of 438 bp and 128 bp, respectively, and were detected by the method described above. The sensitivity of the second product was 48 ag C pneumoniae ATCC VR 2282 cDNA.

Controls

Each DNA extraction and each PCR was accompanied by three negative (control processed together with patient specimens, PCR master mix control for each round) and a positive control (M pneumoniae FH ATCC 15531 kindly provided by K Oberle, Freiburg, Germany and C pneumoniae ATCC VR 2282 kindly provided by M Maass, Lübeck, Germany; fig 1).

All samples were tested for the presence of inhibitors by spiking the samples with pACYC 177 (New England Biolabs, Beverly, MA, USA). This vector has a kanamycin cassette which was detected with the primers Km3 (5'-CAATCAGGTGCGACAATC-3') and Km4 (5'-GCAAGATCC GCA TAT TCG TGA GG-3'), amplifying a 586 bp region of this cassette.

Universal contamination precautions were undertaken to ensure that no amplicon contamination of molecular reagents occurred. They consisted of use of barrier filtered pipette tips, extensive surface cleaning with hypochlorite, and physical separation of extraction and reagent preparations and amplifican detection.

Analysis of data

Statistical analysis was performed with SPSS Version 12.0. Arithmetic means and standard deviation were calculated for continuous data. To calculate statistical significance the Fisher exact test was applied for categorical data and the test for independent continuous data.

RESULTS

Patients

A total of 80 children were enrolled, 26 with asthma, 12 with chronic purulent bronchitis (five with cystic fibrosis, three with primary ciliary dyskinesia, and four with post-infectious bronchiectasis without underlying disease), and 42 control subjects without acute or chronic lung disease. There were no differences in age (p = 0.559 and p = 0.075 for the bronchitis and asthma groups, respectively) or sex (p = 0.77) between patients and controls (table 1). Asthmatics were more likely to be atopic and to be on regular inhaled steroids than patients with chronic bronchitis (p<0.001 for both items). FEV1 was significantly lower in patients with either asthma or chronic bronchitis than in healthy controls (both p<0.001).

Sputum induction

There were no adverse events during or after sputum induction. Induction of adequate amounts of sufficient microscopic quality was possible in 68% of the patients and 57% of the controls (p = 0.24).

Chlamydia pneumoniae

PCR for CP was positive in nine patients (23.6%) with chronic lung diseases and in no patient without lung disease (p = 0.001, table 2). Subgroups of patients with asthma and with chronic bronchitis were significantly more likely to harbour CP within their airways than controls (4/26 v 0/42; p = 0.017 and 5/12 v 0/42; p<0.001, respectively). The difference between CP detection rates in children with asthma (15.3%) and those with chronic purulent bronchitis (41.6%) did not reach statistical significance (p = 0.11). There was an equal detection rate with nasal swabs and induced sputum (13.2% v 19.2%). Of the five patients with nasal specimens positive for CP, only one (the patient with CP in both specimens) had concurrent induced sputum of sufficient quality. When only children with successful sputum induction were analysed, five patients (19.2%) and no controls were positive for CP (p = 0.008). Of the five patients with induced sputum specimens positive for CP, four had negative nasal specimens. Three of nine CP positive patients were also positive for MP in the same specimen. The mean (SD) FEV1 was 95.5 (16.7)% and 90.2 (19.6)% for positive and negative patients, respectively (p = 0.45). 40% of positive and 50% of negative patients were on regular inhaled steroids (p = 1.0). The mean age was 11 years for both CP negative and positive patients (p = 0.54). 50% of non-atopic children and 5.6% of atopic children with asthma were positive for either MP or CP (p = 0.018).

Mycoplasma pneumoniae

10.5% of children with chronic lung disease and none of the healthy controls had evidence of infection with MP (p = 0.044). There were no significant differences in detection rates between asthmatics and patients with chronic purulent bronchitis. No patient had a positive result in both nasal and bronchial specimens. Of the four children with MP colonisation, only one had both nasal and successful sputum induction. If only children with successful sputum induction were analysed, no difference was found in colonisation rates with MP (one patient and no control children; p = 0.42). Three out of four MP positive patients were also positive for

| Table 1 Characteristics of patients at study entry |
|-----------------|-----------------|-----------------|-----------------|
|                 | Asthma (n = 26) | Chronic purulent bronchitis (n = 12) | Healthy controls (n = 42) |
| Mean (range) age (years) | 10.8 (7–15) | 11.8 (7–15) | 12.3 (6–15) |
| Male, n (%) | 12 (46.1%) | 6 (50%) | 24 (57.1%) |
| Atopy, n (%) | 18 (69.2%) | 0 | Not assessed |
| Mean (SD) FEV1 (% predicted) | 94.0 (13.9%) | 86.4 (26.7%) | 109 (10.2%) |
| Bacterial colonisation of sputum | Not assessed | 12 (100%)* | Not assessed |
| Regular systemic or inhaled antibiotics, n (%) | 0 | 12 (100%) | 0 |
| Inhaled steroids, n (%) | 15 (57.8%) | 3 (25%) | 0 |

*Non-typeable Haemophilus influenzae (n = 7), Staphylococcus aureus (n = 3), Pseudomonas aeruginosa (n = 2).
CP in the same specimen. There was no correlation between a positive test result and FEV₁, age, or treatment with inhaled steroids.

**DISCUSSION**

To our knowledge, this is the first controlled study in children with stable asthma and stable chronic purulent bronchitis which shows significantly increased rates of airway colonisation with either CP or MP. The design of our study was cross sectional, so we cannot be sure whether this truly reflects persistence of these organisms. As our patients were free of acute exacerbations, however, it seems unlikely that the differences are merely the result of acute infection. Studies in children and adults suggest that most patients with CP positive nasopharyngeal specimens or sputum remain positive over a time period of at least 1 year. 24 39

Depending on the PCR method used, 1.3–21% of healthy adults 40 and 5–6% of asymptomatic children 41 harbour CP DNA in their nasopharynx or sputum. 44% of lungs after accidental death of previously healthy individuals stained positive for CP antigens. 42 Adequate healthy controls must therefore be included in all studies applying direct pathogen detection techniques for CP in clinical specimens.

Only a few PCR studies have been undertaken in patients with stable chronic lung disease, the majority without adequate controls. In patients with COPD the incidence of CP DNA in spontaneous or induced sputum from patients varied from 11.3% to 59%. 39 43 In adults with asthma Martin and colleagues found DNA of either CP or MP in 56% by using a combination of nasal and oropharyngeal swabs, bronchoalveolar lavage fluid, and bronchial brush and biopsy specimens. 31 In nasopharyngeal swabs CP DNA was detected in 5.4% of adults with acute asthma and 0.9% of healthy controls. 44

Two uncontrolled studies have been performed in children using PCR methods. In a heterogeneous group of children with asthma and recurrent bronchial obstructions, Cunningham et al 32 found a rate of 28% CP DNA but only 3% MP DNA in nasal specimens of stable asthmatic children and showed that PCR for CP remained positive over several months. Schmidt et al 32 reported an incidence of 52% PCR positivity for CP in bronchoalveolar lavage specimens in children undergoing broncoscopic investigation for asthma and recurrent bronchial obstruction. Our study corroborates these findings and also shows that these detection rates are significantly increased when compared with healthy controls. Our study further shows that asthmatic children with atopy were less likely to be colonised with either CP or MP than children without atopy. This is in agreement with findings in adults where onset of asthma in adulthood and non-atopic status were correlated with serological evidence of CP infection. 32

However, the fact that colonisation with either CP or MP occurs in children with asthma or chronic bronchitis, as found in our study, does not establish a causal relationship between infection and inflammation. CP is known to persist within monocytes for a long time without necessarily inducing an inflammatory response. Monocytes and macrophages carry the organism throughout the body 45 and may settle down in loci of increased inflammatory stimuli. Their increased detection rate in children with chronic lung disease with different immunopathology may just reflect the increased concentration of inflammatory cells within respiratory specimens of inflamed airways.

Biscione and colleagues have recently used a reversed transcriptase PCR to detect RNA of the major outer membrane protein (MOMP) from CP that is only produced during productive infection. Nasal secretions were positive for MOMP-RNA in 6.4% of adult patients with stable atopic asthma and in only 2.3% of controls which consisted of the patients’ spouses. 46 This method seems promising for differentiating between colonisation and productive infection.

An intervention trial with antibiotics in patients with repeated PCR measurements of CP and MP in respiratory specimens will be necessary to clarify whether CP or MP persist in inflamed airways as a pathogenic co-factor or as an innocent bystander. A recent randomised controlled trial of roxithromycin given for 6 weeks to adults with asthma and serological evidence for CP infection showed a short term increase in evening peak flow but no long term effect. 33 Another two uncontrolled studies have been performed in patients with positive CP PCR. Blasi et al 39 demonstrated microbiological success, defined as clearance of CP DNA from blood monocytes, in 59% of adults with a COPD exacerbation after a 6 week course of azithromycin which was sustained 10 weeks after the treatment in only 29%. In the second uncontrolled study Miyashita et al 39 reported that clarithromycin given for up to 6 weeks to adult patients with chronic cough eradicated CP from nasal swab specimens and stopped symptoms.

One small controlled study by Kraft et al 49 in 55 adults with asthma used direct pathogen detection in bronchoalveolar lavage fluid and bronchial biopsies as the entry criterion. In this study clarithromycin improved FEV₁ and decreased concentrations of TNF-α, IL-12 and IL-5 compared with placebo only in those asthmatics who were PCR positive for either MP or CP, which suggests that macrodides have a specific antimicrobial effect as well as anti-inflammatory activity. Because of the invasive nature of CP and MP detection techniques in this study, microbiological success could not be documented by obtaining repeated respiratory specimens during and after the intervention.

Before starting intervention trials PCR detection methods should be replicated in larger numbers of patients and within a longitudinal study. If colonisation with CP or MP can be detected repeatedly in the same patients, combining nasal brush with induced sputum specimens (as in our study) would be a feasible method for measuring microbiological success of antibiotic interventions trials. Performing these trials in children with asthma and other chronic lung diseases would be worthwhile as there is evidence from

| Table 2 Number (%) of patients who tested positive for Mycoplasma pneumoniae and Chlamydophila pneumoniae in nasal brush specimens or induced sputum |
|-----------------|-----------------|-----------------|
|                  | Chlamydophila pneumoniae | Mycoplasma pneumoniae |
|                  | Nasal brush | Induced sputum | Nasal brush | Induced sputum |
| Asthma           | 2 (7.6%)    | 3 (13%)     | 2 (7.6%)    | 1 (5%)       |
| Chronic purulent bronchitis | 3 (25%)   | 2 (33%)     |             |              |
| Healthy controls | 0           | 0           | 0           | 0            |
animal studies that early treatment may be more effective than later treatment.15

ACKNOWLEDGEMENTS
The authors thank K Oberle, Freiburg, Germany for providing M pneumoniae FH ATCC 15531 and M Maas, Liibeck, Germany for providing C pneumoniae ATCC VR 2282 as positive controls; and Karin Kogelheine, technical assistant at Children’s Hospital Bochum, for performing lung function tests and sputum cytology as well as supporting sputum induction procedures.

Authors’ affiliations
N Teig, C Schmidt, C Rieger, Children’s Hospital of Ruhr University, St Josef Hospital, Bochum, Germany
A Anders, S Gatermann, Institute of Microbiology, Immunology and Hygiene, Ruhr University, Bochum, Germany

The study was supported by a grant from the medical faculty (FoRM grant) of the Ruhr-University Bochum.

Competing interest: None of the authors has any financial interests in any of the diagnostic tools used in the study.

REFERENCES
Chlamydia pneumoniae and Mycoplasma pneumoniae in respiratory specimens of children with chronic lung diseases

N Teig, A Anders, C Schmidt, C Rieger and S Gatermann

Thorax 2005 60: 962-966 originally published online September 2, 2005
doi: 10.1136/thx.2005.041004

Updated information and services can be found at:
http://thorax.bmj.com/content/60/11/962

These include:

References

This article cites 47 articles, 16 of which you can access for free at:
http://thorax.bmj.com/content/60/11/962#BIBL

Email alerting service

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections

Articles on similar topics can be found in the following collections

Asthma (1782)
Child health (843)
Bronchitis (235)
TB and other respiratory infections (1273)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/