

## PAEDIATRIC LUNG DISEASE

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

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**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.

*Chlamydophila pneumoniae* (CP) and *Mycoplasma pneumoniae* (MP) are intracellular organisms which primarily infect ciliated epithelial cells and alveolar macrophages.<sup>1,2</sup> They are considered to be frequent causes of community acquired pneumonia in school children.<sup>3</sup> Serological studies have shown that 64% of all children have had at least one infection with CP during their first 8 years of life.<sup>4</sup> During acute infection both organisms evoke an immunopathological reaction and can cause bronchial hyperresponsiveness,<sup>5,6</sup> both of which are important for the pathogenesis of chronic airway diseases. Both organisms are considered to be trigger factors for asthma and chronic obstructive pulmonary disease (COPD) exacerbations.<sup>7,8</sup>

CP and, to a lesser degree, MP have the propensity to persist within several cell types<sup>9,10</sup> and animals.<sup>11</sup> Seroepidemiological studies in adults have linked persistent CP infection to asthma<sup>12-16</sup> and COPD,<sup>17,18</sup> as well as to several extrapulmonary chronic diseases.<sup>19-21</sup>

Conflicting results have been reported from seroprevalence studies in children with asthma.<sup>22,23</sup> One study performed in children with asthma found an association between asthma severity and titres of specific secretory IgA from nasal secretions.<sup>24</sup> Seroprevalence rates of CP antibodies in children with asthma were found to be increased in one study<sup>22</sup> but not in another.<sup>23</sup> Serological methods, however, are neither sensitive nor specific for the chronicity and the actual presence or activity of these organisms.<sup>25</sup> All tests available may show cross reactivity with other chlamydial species or lipopolysaccharides from other bacteria,<sup>26</sup> and the correlation between polymerase chain reaction (PCR) positivity and serological positivity is generally poor. PCR has therefore been proposed to be the method of choice to clarify the role of persistent CP and MP infections in chronic lung diseases.<sup>27</sup> Animal and cell culture studies suggest that persistent

infection with CP occurs at a low replicative state.<sup>28</sup> Culture techniques for MP or CP are insensitive even in cases of acute infection when compared with DNA based techniques<sup>29,30</sup> and are not therefore suitable for the diagnosis of persistent infection.

There are few studies using PCR techniques in asthmatics without acute exacerbations. Only one study done in adults with chronic asthma included an appropriate control group.<sup>31</sup> In this study MP and, to a lesser degree, CP were detected in a significant proportion of patients but most patients were only positive in bronchial biopsy specimens, precluding the use of this method on a large scale basis. Two other uncontrolled studies performed in children with asthma reported positive results for CP DNA in 25% of nasal aspirates<sup>34</sup> and bronchoalveolar lavage specimens.<sup>32</sup>

As early (but not late) antibiotic treatment of experimental CP infection protects animals from developing atheromatous plaques,<sup>33</sup> information on the age at which chronic infection can be detected may be important for intervention studies. We therefore investigated the role of both organisms in school children with stable chronic lung diseases (asthma, chronic purulent bronchitis) and children without lung disease by PCR analysis of induced sputum and nasal brush specimens.

## METHODS

### Patients

Children aged 6-16 years with either asthma or chronic purulent bronchitis and controls without lung disease were enrolled prospectively in several outpatient clinics of our

**Abbreviations:** CP, *Chlamydophila pneumoniae*; FEV<sub>1</sub>, 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 over more than 2 years and at least two positive sputum cultures for conventional bacteria within 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 *Chlamydomphila* 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 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 (FEV<sub>1</sub>) 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.<sup>34</sup> 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 300g 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 (10<sup>5</sup>–10<sup>6</sup> viable cells/ml) in >96% of specimens obtained from healthy school children.

### Detection of *Mycoplasma pneumoniae* and *Chlamydomphila pneumoniae* DNA by nested PCR

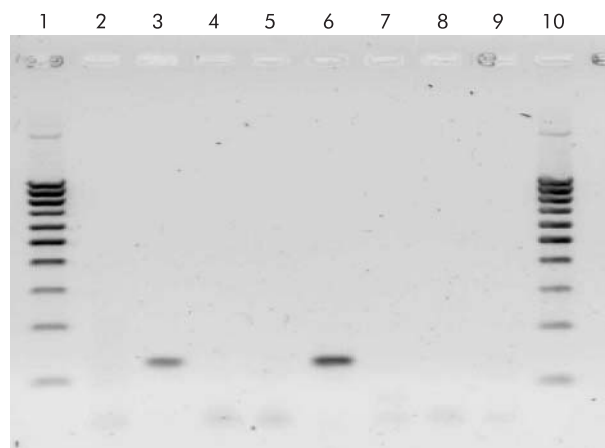
The laboratory investigators were blind to the patients' data.

#### *Mycoplasma pneumoniae*

A total of 500 µl nasopharyngeal brush specimen or induced sputum submitted from the Children's Hospital was pre-treated 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 TAT GGT ACA GGT TGG 3' and MP-2 5'-ATT ACC ATC CTT GTT GTA ACG-3' (MWG-Biotech, Ebersburg, Germany) described by Bernet,<sup>35</sup> 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 CTG GAA GGA TGT TAA GC-3' and MUH-2 5'-TTTG TAA TCG TCT TTA TTT CG-3'.<sup>36</sup>

Amplification reactions were performed in a volume of 50 µl containing 10 × PCR buffer with 1.5 mM MgCl<sub>2</sub> (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° 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 ag *M pneumoniae* FH ATCC 15531 cDNA.



**Figure 1** *Chlamydomphila pneumoniae* nested PCR. Lanes 1 and 10, 100 bp DNA ladder; lanes 2–5, clinical samples; lane 6, positive control; lanes 7–9, negative controls. Lanes 3 and 6 show positive 128 bp PCR products for *C pneumoniae*.

### *Chlamydomydia 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 GTT CAT GAA GGC CTA CT-3' and CpHR-1 5'-TGC ATA ACC TAC GGT GTG TT-3' which is specific for CP<sup>35</sup> and the inner primer pair CpIn-1 5'-AGT TGA GCA TAT TCG TGA GG-3' and CpIn-2 5'-TTT ATT TCC GTG TCG TCC AG-3'.<sup>37</sup> These primers amplify a sequence of unknown function of CP and do not amplify DNA from normal flora or any other respiratory pathogen.<sup>38</sup>

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 TGGTATCGG-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 amplification 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 two sided *t* 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). FEV<sub>1</sub> 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 ( $p = 0.24$ ).

### *Chlamydomydia 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) FEV<sub>1</sub> 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 sex, n (%)	12 (46.1%)	6 (50%)	24 (57.1%)
Atopy, n (%)	18 (69.2%)	0	Not assessed
Mean (SD) FEV <sub>1</sub> (% 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).

**Table 2** Number (%) of patients who tested positive for *Mycoplasma pneumoniae* and *Chlamydomphila pneumoniae* in nasal brush specimens or induced sputum

	<i>Chlamydomphila pneumoniae</i>		<i>Mycoplasma pneumoniae</i>	
	Nasal brush	Induced sputum	Nasal brush	Induced sputum
Asthma	2 (7.6%)	3 (15%)	2 (7.6%)	1 (5%)
Chronic purulent bronchitis	3 (25%)	2 (33%)	1 (8.3%)	0
Healthy controls	0	0	0	0

CP in the same specimen. There was no correlation between a positive test result and FEV<sub>1</sub>, 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.<sup>24–39</sup>

Depending on the PCR method used, 1.3–21% of healthy adults<sup>40</sup> and 5–6% of asymptomatic children<sup>30–41</sup> harbour CP DNA in their nasopharynx or sputum. 44% of lungs after accidental death of previously healthy individuals stained positive for CP antigens.<sup>42</sup> 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%.<sup>39–43</sup> 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.<sup>31</sup> In nasopharyngeal swabs CP DNA was detected in 5.4% of adults with acute asthma and 0.9% of healthy controls.<sup>44</sup>

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*<sup>24</sup> 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*<sup>22</sup> reported an incidence of 52% PCR positivity for CP in bronchoalveolar lavage specimens in children undergoing bronoscopic 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.<sup>12</sup>

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<sup>45</sup> 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.<sup>46</sup> 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.<sup>47</sup> Another two uncontrolled studies have been performed in patients with positive CP PCR. Blasi *et al*<sup>39</sup> 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*<sup>48</sup> 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*<sup>49</sup> 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 FEV<sub>1</sub> and decreased concentrations of TNF- $\alpha$ , IL-12 and IL-5 compared with placebo only in those asthmatics who were PCR positive for either MP or CP, which suggests that macrolides 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

animal studies that early treatment may be more effective than later treatment.<sup>33</sup>

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