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Chronic *Chlamydia pneumoniae* infection and asthma exacerbations in children

A.F. Cunningham*, S.L. Johnston+, S.A. Julious§, F.C. Lampe§, M.E. Ward*

Chronic Chlamydia pneumoniae *infection and asthma exacerbations in children. A.F. Cunningham, S.L. Johnston, S.A. Julious, F.C. Lampe, M.E. Ward. ©ERS Journals Ltd 1998.*
ABSTRACT: This study was undertaken to investigate the reported association between *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* infection and the expression of asthma-related symptoms.

One hundred and eight children with asthma symptoms, aged 9–11 yrs, completed a 13 month longitudinal study. The children maintained a daily diary of respiratory symptoms and peak flow rates. When respiratory symptoms were reported an investigator was called and a nasal aspirate obtained. In total 292 episodes were reported. After the study 65 children provided samples when asymptomatic. The presence of infection was investigated by the polymerase chain reaction for *C. pneumoniae* and *M. pneumoniae* and *C. pneumoniae* secretory immunoglobulin A (IgA) was detected by amplified enzyme immunoassay.

***C. pneumoniae* detections were similar between the symptomatic and asymptomatic episodes (23 *versus* 28%, respectively). Children who reported multiple episodes also tended to remain PCR positive for *C. pneumoniae* suggesting chronic infection (p< 0.02). *C. pneumoniae*-specific secretory-IgA antibodies were more than seven times greater in subjects who reported four or more exacerbations in the study compared to those who reported just one (p<0.02). *M. pneumoniae* was found in two of 292 reports and in two of 65 asymptomatic samples.**

In conclusion, chronic *Chlamydia pneumoniae* infection is common in schoolage children and immune responses to *C. pneumoniae* are positively associated with frequency of asthma exacerbations. We suggest that the immune response to chronic *C. pneumoniae* infection may interact with allergic inflammation to increase asthma symptoms. In contrast *Mycoplasma pneumoniae* was not found to be important in this study.
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*Molecular Microbiology, +University Medicine and §Dept of Medical Statistics and Computing, Southampton University Medical School, Southampton General Hospital, Southampton, UK.

Correspondence: A. Cunningham
Dept of Infection
University of Birmingham
Edgbaston
Birmingham B15 2TT
UK
Fax: 44 1214146956

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Recent studies have highlighted the importance of viral upper respiratory tract infections in acute exacerbations of asthma in both adults and children [1–3]. Previous work suggests that common bacterial infections do not play a role in acute exacerbations [1]. However, the role of atypical bacteria such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* in asthma has not been sufficiently investigated.

C. pneumoniae is a common respiratory pathogen, and although many infections are believed to be asymptomatic [4–7], *C. pneumoniae* infection is related to a broad spectrum of diseases, such as atypical pneumonia, heart disease and asthma [8–13]. The first association between asthma and *C. pneumoniae* was described by HAHN *et al.* [10], who reported wheezing in nine of 19 adult patients with serological evidence of current or recent *C. pneumoniae* infection. A second study [13] found 11% of asthmatic and 5% of control children to be culture positive for *C. pneumoniae*. Further evidence for a possible role for *C. pneumoniae* comes from the observation from uncontrolled studies that some subjects treated with antichlamydial antibiotics reported improvements in their asthma symptoms [11, 13].

M. pneumoniae is the causative agent in a number of respiratory diseases including upper respiratory tract illnesses such as rhinitis, pharyngitis or otitis, as well as bronchitis and atypical pneumonia. *M. pneumoniae* has been sought in previous studies of asthma exacerbations in children and was detected, on average, in 2–3% of cases [1]. In the sole study in adults, the detection rate was 3.5% [14].

Taken together, these studies suggest that *C. pneumoniae* and/or *M. pneumoniae* may be important in acute exacerbations of asthma in adults and children. However, previous studies were hampered by suboptimal detection techniques [15, 16].

The purpose of this study was to investigate the role of these organisms in acute exacerbations of asthma. To achieve this aim we applied sensitive detection techniques to samples collected during an intensive prospective clinical study of asthma exacerbations in schoolage children.

Materials and methods

Subject criteria and sample collection

Between April 1989 and April 1990, 108 children aged 9–11 yrs, who had previously responded positively to a

questionnaire enquiring into the presence of asthma symptoms, (cough and/or wheeze), maintained diary cards of upper and lower respiratory symptoms and peak expiratory flow (PEF). The investigator was notified in the event of a fall in PEF of ≥50 $L \cdot min^{-1}$ and/or an episode of respiratory symptoms. A nasal aspirate and acute and convalescent sera were obtained at each of 292 reports made by 96 children, with a median fall in PEF of 80 $L \cdot min^{-1}$ (26% fall from baseline). Control asymptomatic aspirates were obtained from 65 of the same children during July and August 1991. Of the 108 children, 62 (57%) were atopic, 42 (39%) had physician diagnosed asthma, 18 (17%) were taking prophylactic inhaled steroids or disodium cromoglycate, three (3%) took regular theophyllines and 21 (19%) took regular or as required inhaled β-agonists. Fifty eight (54%) of the enrolled children were boys. Full details of the cohort, methods of surveillance and reported episodes have been published previously [2].

Deoxyribonucleic acid (DNA) extraction

One hundred microlitres of nasal aspirate was digested by proteinase K (50 $\mu g \cdot mL^{-1}$) for 3 h at 55°C, phenol extracted, ethanol precipitated and then resuspended in distilled water. Negative controls, of water only, were incorporated into each batch of 22 extractions.

Primers and C. pneumoniae *polymerase chain reaction (PCR) conditions*

Nested primers were selected from major outer membrane protein (MOMP) omp1 alignments of published *C. pneumoniae* IOL207, *C. trachomatis*, and *C. psittaci* sequences, as shown in figure 1. Reactions contained 1× PCR buffer, each primer at a final concentration of 1 µM (table 1), 0.8 mM deoxyribonucleotide triphosphates (dNTPs), 1 U of Taq DNA polymerase (Promega, Southampton, UK) and 3 µL of template. DNA was amplified for 30 cycles on a Perkin Elmer Cetus model 480 DNA Thermal Cycler (Applied Biosystems Ltd., Warrington, UK). Denaturation was at 94°C for 1 min and extension at 72°C for 1.5 min. Annealing times were 1 min at 52°C for APNOL and APNOU (primary amplification) and 63°C for APN1 and APN2 (for nested amplification). A positive result yielded a 488 base pair (bp) product. A short (550 bp) cloned fragment of *C. pneumoniae* genomic DNA and water were used as positive and negative controls respectively. All test and control nasal aspirates were tested blindly. The nested

APNOU	5'AATTCTCTGTAAACAAACCC3'	*C. pneumoniae* IOL207
	5'★★★★TGTGA★TC★★★★★★A3'	*C. psittaci* MN
	5'★G★★TA★★A★T★★T★★★★★T3'	*C. trachomatis* L1
APNOL	5'ATTAAGAAGCTCTGAGCATA3'	*C. pneumoniae* IOL207
	5'★CCCG★★GAGA★CT★★GT★C3'	*C. psittaci* MN
	5'GCCC★★★★A★A★G★★TAG★G3'	*C. trachomatis* L1
APN1	5'TGCCAACAGACGCTGGCGT3'	*C. pneumoniae* IOL207
	5'★A★★T★T★ACG★★★★★AAC3'	*C. psittaci* MN
	5'★TGATCTTACA★★A★★AAC3'	*C. trachomatis* L1
APN2	5'AGCCTAACATGTAGACTCTGAT3'	*C. pneumoniae* IOL207
	5'TT★★★★GGT★C★GATAG★G★GA3'	*C. psittaci* MN
	5'★AAGAT★A★CT★GCCAAAGTT★3'	*C. trachomatis* L1

Fig. 1. – Analysis of alignments to the four primers. *C.*: *Chlamydia*.

Table 1. – Primer sequences and details

Primer	Site bp*	Sequence 5'→3'	Direction
APN1	737	TGCCAACAGACGCTGGCGT	Sense
APN2	1223	AGCCTAACATGTAGACTCTGAT	Antisense
APNOU	689	AATTCTCTGTAAACAAACCC	Sense
APNOL	1249	ATTAAGAAGCTCTGAGCATA	Antisense
T4APN4L	1103	GACCATTTATCAGCATCAAC	Antisense
4APN10U	842	TAGTGCCATACATTGGAGTA	Sense

*: The distance downstream from the *omp1* start codon in bases of the primers first 5' base. bp: base pairs.

PCR could detect 5 fg of *C. pneumoniae* chromosomal DNA, equivalent to approximately five genome equivalents. No cross reactivity was observed between the primers and DNA from *C. trachomatis* and *C. psittaci*. All positive samples were confirmed three times, re-extracted from a second, separate aliquot where possible (n=40), and amplified from a region of the chromosome not incorporated in the cloned PCR positive control (n=51) using alternative sets of primers. All DNA extractions, primary PCR amplifications and secondary PCR amplifications were undertaken in separate areas, using dedicated pipettes and filter tips. A minimum of four negative controls were incorporated into each PCR run.

PCR detection of Mycoplasma pneumoniae

The methods and primers used for the detection of *M. pneumoniae* by nested PCR were those of NARITA *et al*. [17] and generated a 108 bp fragment. Positive controls were *M. pneumoniae* DNA (PHLS, Colindale, UK), and negative controls were water only.

Detection of C. pneumoniae*-specific secretory component in nasal aspirates*

Local antibody responses were detected by the ELAST amplified enzyme immunoassay technique, (NEN Life Science Products, Hounslow, UK) as per the manufacturer's instructions. This technique has been used to study the epidemiology of *C. trachomatis* [18]. Briefly, plates were coated with 10 $\mu g \cdot mL^{-1}$ purified *C. pneumoniae* (VR1310) organism, and blocked with 1% bovine serum albumin (BSA) (Sigma, Poole, UK). Primary antibody (1:100) was incubated at 37°C for 3 h or overnight at 4°C. Conjugate antibody (horseradish peroxidase labelled anti-human secretory-piece; Dako, High Wycombe, UK; 1:1000) was added for 1 h at 37°C. After primary conjugation the amplification steps were undertaken. Biotinyl-tyramide working solution was added and the plates incubated at room temperature for 20 min before the streptavidin-horseradish peroxidase secondary conjugate was added. Trays were then incubated at room temperature for a further 30 min and absorbances read at 450 nm.

DNA sequencing

Positive PCR reactions for *C. pneumoniae* were confirmed by sequencing of the product. Template DNA was initially amplified by PCR, using the *C. pneumoniae* primers and conditions described above, for forty cycles.

These products were purified using the Geneclean™ kit (Anachem, Luton, UK) and sequenced using the primers 4APN10U and T4APN4L (table 1) on an Applied Biosystems Ltd (Warrington, UK) 373A Automated Sequencer using the Applied Biosystems Ltd. Taq Dye Deoxy™ Terminator Cycle protocol with reaction conditions of 96°C/15 s, 45°C/10 s and 60°C/min for 25 cycles.

Statistical analysis

Analysis of results was undertaken using the Statistical Products and Service Solutions (SPSS) package (SPSS; Chicago, IL, USA) [19]. The Kruskal-Wallis χ^2 nonparametric analysis of variance was used to investigate the relationship between frequency of reported episodes and the mean local antibody response for each child. To investigate the effect of increasing adjusted absorbance on risk of being PCR positive, a logistic regression was underta-ken with PCR positivity at each report as the dependent variable and adjusted absorbance quartiles as the indepen-dent variable. A random subject term was also included in the model using a random effects logistic regression model in the EGRET statistical package (Statistics and Epide-miology Research Co. and Cytel System Co., Seattle, WA, USA) [20]. This was done to correct for the within subject correlation of repeated adjusted absorbance readings recorded on individual subjects. The change in the -2×log-likelihood χ^2 was used to assess significance.

The Pearson χ^2 test was used to assess whether subjects PCR positive on any report were more likely to be positive on their next report. The relationship between PCR positivity on first report and the total number of subsequent PCR positives was assessed using the Mann-Whitney test.

Results

PCR analysis of nasal aspirate samples

Positivity rates. When samples from the reported symptomatic episodes were tested by nested PCR, 43 out of 96 (45%) of the children who provided specimens during the study were positive for *C. pneumoniae* on at least one occasion during the study. From the 292 reported symptomatic episodes, 68 out of 292, (23%), were positive for *C. pneumoniae* compared with 18 out of 65 (28%) of the samples collected when asymptomatic 15 months after the completion of the study. Of the children who were PCR positive for *C. pneumoniae* in the asymptomatic samples, seven out of 18 (39%) had been PCR positive during the study.

Chronicity of infection. Subjects found to be PCR positive in one reported episode were likely to remain PCR positive when they next reported (Pearson χ^2=6.081, degrees of freedom (df)=1, p=0.014). The likelihood of chronic *C. pneumoniae* infection was such that 35% of subjects who were PCR positive on a particular report remained so on their next reported episode, compared to 18% of *C. pneumoniae* negative subjects. Furthermore, if a subject was PCR positive on their first reported episode then that subject was significantly more likely to be PCR positive on any subsequent reported episode (Mann-Whitney z= 2.061, p=0.009). These findings suggest that *C. pneumoniae* infection was chronic.

Characteristics of illness. *C. pneumoniae* has been associated with a biphasic illness pattern. Thus, the symptoms recorded in the daily diary records were compared 5–7 weeks before their first PCR positive and 5–7 weeks after this report. Of the 43 children who were PCR positive at least once during the study, these data could be analysed for 24 of them. The other 19 were excluded due to the 5–7 week period extending either before or after the study had started or ended. It was noted that there was a greater likelihood that a child would experience lower respiratory tract symptoms 5–7 weeks after their first PCR positive than before (p=0.06). Similar findings have been reported elsewhere [21].

Verification of sample identity by direct PCR sequencing

To confirm the PCR results, a number of PCR products (14 reported samples and four asymptomatic), were chosen at random and sequenced. Sequences were identical to those published for *C. pneumoniae* IOL207 [22]. No sequence variants were observed.

Role of other pathogens

These samples had been tested previously for all common respiratory viral pathogens, and 80–85% of exacerbations were associated with positive viral infections [2]. In 18 of the 68 (27%) of *C. pneumoniae* PCR diagnosed infections no other pathogen was identified; in 46 of the 68 (68%) one other pathogen was identified and in four of the 68 (6%) two other pathogens were identified.

Detection of local C. pneumoniae*-specific antibody response by amplified enzyme immunoassay*

Two hundred and sixty nine out of 292 respiratory secretions were available for testing for secretory antibody to *C. pneumoniae* using a sensitive, amplified enzyme immunoassay (EIA). Samples were tested in duplicate for secretory immunoglobulin A (IgA). EIA background for each tray was taken as the mean plus two standard deviations of the lower quartile of all absorbances. This value was then subtracted from the duplicate means of the test samples to give absorbance above background, this was termed adjusted absorbance.

The number of episodes reported by each child during the 13 months of the study was used to assess the relationship between asthma exacerbations and local antibody to *C. pneumoniae*. The mean adjusted absorbance of all episodes for each individual child was compared between children making a total of 1, 2, 3, 4 and ≥5 reports. There was a significant trend of increasing adjusted absorbance with increasing number of reports made. Medians of the mean adjusted absorbance in groups making 1, 2, 3, 4 and ≥5 reports were 0.006, 0.009, 0.027, 0.048 and 0.043 respectively (Kruskall-Wallis χ^2=11.770, df=4, p=0.019) (fig. 2).

Detection of Mycoplasma pneumoniae

Only two out of 292 (1%) of the report samples were positive for *M. pneumoniae* by PCR (one of which was a co-infection with *C. pneumoniae*. Two out of 65 (3%) of the asymptomatic samples were also positive for *M. pneumoniae* by PCR.

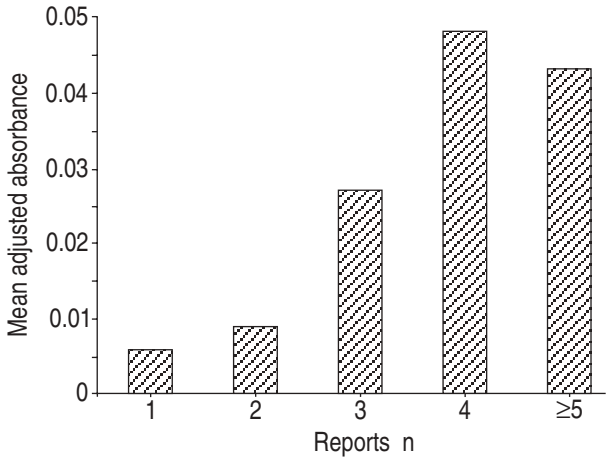

Fig. 2. – Relationship between mean adjusted absorbance of anti-*Chlamydia pneumoniae* secretory immunoglobulin A and the number of episodes reported by children. Reports are expressed as number per child and mean adjusted absorbance is defined in the text.

Discussion

We report an unexpectedly high prevalence of low grade *C. pneumoniae* infection in this schoolage cohort of children with asthma symptoms. We believe that the use of a highly sensitive nested PCR, a technique considered more sensitive than culture [23], performed on nasal aspirates rather than swabs [24], contributed to the outcome. It is clear that infection was of a low grade since a single step PCR, which was twenty times less sensitive only detected three of the infections identified by the nested PCR [25]. Also, of the 68 PCR positive episodes, 50 were positive for at least one other pathogen suggesting that *C. pneumoniae* co-infections are common. The possibility of false positive detection of *C. pneumoniae* as a result of contamination was avoided by the use of stringent precautions including separate working areas for the pre- and post-PCR amplification stages, the use of separate pipettes with filter tips, the nucleotide sequencing of PCR products and independent confirmation of positive results from previously unopened aliquots of the specimen.

In those children where *C. pneumoniae* was detected by PCR, the infection tended to be chronic, as positive children were more likely than negative children to be positive in subsequent reported episodes. Although it is assumed that these *C. pneumoniae* infections were chronic it is impossible, in the absence of a reliable strain typing procedure, to eliminate the possibility that some children were frequently re-infected. However, this is unlikely as chronic infection has previously been reported for both *C. pneumoniae* and *C. trachomatis* and appears to be common in the course of chlamydial infections [5, 26].

Nucleotide sequencing of the variable segment 4 region of the omp1 gene failed to reveal any heterogeneity in this region. This finding is in agreement with that of GAYDOS *et al*. [27] and indicates that *C. pneumoniae*, unlike *C. trachomatis*, shows no sequence variability in this segment of the gene. This lack of variation may be because the *C. pneumoniae* MOMP, encoded by omp1, unlike the *C. trachomatis* MOMP, appears not to be immunodominant [28].

M. pneumoniae was not found to be an important cause of respiratory morbidity in this study. *M. pneumoniae* in the UK has an epidemic cycle of approximately 5 yrs and the reported episode samples were collected during a trough in this cycle.

Retrospective population seroprevalence studies using microimmunofluorescence indicate that lifetime exposure rates for children of this age are about 10–30% [8]. However, not all *C. pneumoniae* infections provoke a humoral immune response detectable by microimmunofluorescence [29], and some isolation studies from subjects with airway dysfunction have found rates higher than expected on the basis of serological data [13]. This suggests that the subjects in this study have a higher prevalence than normal children, that serological studies have underestimated the true prevalence of this organism, or both.

The finding of increased numbers of asthma exacerbations in those children with higher local antibody responses to *C. pneumoniae* is intriguing and suggests that *C. pneumoniae* infection may be more prevalent, and/or the local antibody responses to *C. pneumoniae* may be greater or qualitatively different, in subjects with more severe asthma. We were unable to address these questions in this study as it was carried out on samples collected during a study on the role of viral infections in acute exacerbations of asthma [2] and during the design of the study, the data generated by these investigations was not anticipated. In addition, the nature of the study was very arduous for the children undertaking it, and it was thought unlikely that a control population of normal children would provide matched commitment to the study. Serum antibody responses to *C. pneumoniae* were not assessed, as insufficient serum was available from the original study to allow these investigations to be undertaken.

This study found local antibody responses to *C. pneumoniae* that were over seven times greater in subjects who reported four or more symptomatic episodes during the study than in subjects who just reported one episode, yet no evidence for an association between acute *C. pneumoniae* infection and acute exacerbations of asthma was found. However, chronic *C. pneumoniae* infection was common. Considering this association between the immune response to *C. pneumoniae* and asthma symptom frequency it seems logical to suggest that chronic *C. pneumoniae* infection may act as a cofactor, possibly rendering the subject more susceptible to other stimuli such as viruses or allergens or both. Another possible explanation is that the *C. pneumoniae*-specific secretory IgA was raised as part of a polyclonal increase in immunoglobulin synthesis in those children with more frequent exacerbations. Unfortunately, we were unable to test this latter hypothesis because of a shortage of remaining clinical material. However, the confirmation of the presence of *C. pneumoniae* by PCR in this population makes this explanation less likely. It has been reported that type 2 T-helper (Th2) lymphocyte responses may play a role in the immunopathology of ocular *C. trachomatis* infection in trachoma [30], and Th2 responses are thought to play an important role in the inflammatory mechanisms of asthma [31]. We therefore hypothesize that *C. pneumoniae* infection in asthma may promote allergic inflammation by increasing pro-inflammatory Th2 type responses in susceptible individuals. Thus the immunological response to *C. pneumoniae* infection may be related to its pathogenicity.

This hypothesis is supported by a recent study, which suggested that there may also be a relationship between *C. trachomatis* infections, as detected by microimmunofluorescence, asthma-related symptoms and bronchial hyperresponsiveness [32].

In conclusion, this study reports an unexpectedly high prevalence of chronic *Chlamydia pneumoniae* infection in a cohort of children with asthma symptoms, and increased reporting of asthma exacerbations in those children with the greatest local antibody response to *C. pneumoniae*. These findings suggest a possible role for chronic *C. pneumoniae* infection in childhood asthma. The hypothesis that chronic *C. pneumoniae* infection is implicated in increased asthma symptoms should be tested in further studies.

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