Pediatr Allergy Immunol 2005: 16: 145–150. DOI: 10.1111/j.1399-3038.2005.00244.x
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PEDIATRIC ALLERGY AND IMMUNOLOGY

Why *Chlamydia pneumoniae* is associated with asthma and other chronic conditions? Suggestions from a survey in unselected 9 yr old schoolchildren

Ronchetti R, Biscione GL, Ronchetti F, Ronchetti MP, Martella S, Falasca C, Casini C, Barreto M, Villa MP. Why *Chlamydia pneumoniae* is associated with asthma and other chronic conditions? Suggestions from a survey in unselected 9 yr old schoolchildren.
Pediatr Allergy Immunol 2005: 16: 145–150. © 2005 Blackwell Munksgaard

Despite numerous studies demonstrating an association between asthma and many other chronic conditions and signs of *Chlamydia pneumoniae* (Cp) infection, the role of Cp in the pathogenesis of these illness remain still unclear. We investigated the prevalence of Cp antigen in the upper airways and the prevalence of detectable Cp serum antibodies in an unselected population of 207 9-yr-old schoolchildren. We also sought the presence of asthma, chronic or recurrent respiratory symptoms by means of questionnaire completed by the parents. Nasal aspirate, blood sampling and allergen skin prick tests were also performed. None of the children had obvious signs of acute infection at physical examination. Cp DNA was detected in nasal aspirates from 20 of the 207 children tested and serum IgG antibodies for Cp in 68 children. No association was found between atopy or history of atopic illness and the presence of Cp DNA or antibody production. This finding is explained by the fact that our study was conducted in an unselected childhood population, inherently including few children with asthma. A strong association between the status of antigen carrier and the presence of detectable Cp serum immunoglobulin (Ig)G or IgM suggests that subjects with detectable Cp antibodies have an impaired ability to eliminate this pathogen when infected. Because Cp eradication requires a strong Th1 lymphocyte response, the previously proven association between Cp and asthma, might reflect the known association of asthma with Th2-oriented lymphocytic activity.

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Key words: serum antibodies; *Chlamydia pneumoniae*; DNA; infection; asthma; atopy; children

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Accepted 3 November 2004

Clamydia pneumoniae (Cp) is a common cause of acute respiratory tract infections (1). Adult infections caused by this agent may be long-lasting and often chronic (2–3); Cp has also been found in association with asthma (4) but also with chronic diseases, including atherosclerosis (5–6), chronic obstructive pulmonary disease (COPD) (4), ischemic hearth disease (7–8), colesteatoma (9) and sudden infant death syndrome (10).

In particular the role of Cp in the pathogenesis of asthma (11), has been the target of a growing interest, but the relationship has never been investigated, to our knowledge, in an unselected population of children. The present situation is that despite so many studies it is not clear if Cp is simply associated with all those clinical conditions or if there is clear evidence that Cp is actively and directly responsible at least in part in the pathogenesis of them.

This study was designed to find epidemiological support for one or the other of these two hypotheses. We investigated the prevalence of Cp DNA in the upper airways and the prevalence of

detectable Cp serum antibodies in an unselected population of schoolchildren from Central Italy. We also sought the presence of asthma, chronic or recurrent respiratory symptoms by means of questionnaire and of atopy by means of allergen skin prick test.

Material and methods

Study population

The population was recruited by sending a letter to all parents of children from classes IV and V (9–11 yr of age) at an elementary school in Ronciglione (Viterbo) in Central Italy. Parents were asked to complete a questionnaire and to give permission for their children to be tested. Of the 240 parents interviewed, 19 refused permission for blood sampling or skin testing; 14 children did not participate in the study for other reasons. The remaining 207 subjects were included in the study and underwent nasal aspirate, blood sampling and allergen skin prick tests.

At the moment of the study no child had major signs of acute respiratory infection. The study was approved by the Ethical Committee of the Pediatric Clinic Department of the University 'La Sapienza' of Rome.

Questionnaire

Parents completed a modified version of the American Thoracic Society questionnaire for respiratory symptoms (12) seeking information on family history (health and cigarette consumption) and the child's history of respiratory symptoms (ever and in the past 12 months). Our questionnaire has been described in detail elsewhere(13–14).

Allergy assessment

Skin allergen sensitization was measured by the mean wheal size of skin prick tests on the volar aspect of the forearm. Allergens comprised *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat hair, molds (*Aspergillus fumigatus* and *Alternaria tenuis*), mixed grass, mixed tree pollen, and *Parietaria officinalis* (Soluprick; ALK-Abellò, Horsholm, DK). Histamine dihydrochloride (10 mg/ml) and diluent (50% glycerol and 50% physiological saline) were tested as positive and negative controls. Wheal size was recorded as the long average of the axis and its perpendicular. A wheal of ≥3 mm in size was considered as positive. Atopy was defined as the presence of at least one allergen positive skin reaction. The sum of all positive allergen reactions (excluding *D. farinae*), was defined as the 'prick index' (15).

Nasal aspirates

Nasal aspirate samples were obtained by suction with a soft tube filled with sterile, room-temperature normal saline solution following the technique described by Johnston et al. (16). The aspirates were stored at −70°C until further testing by polymerase chain reaction (PCR).

DNA PCR Method

One hundred microliter of nasal aspirates was proteinase K digested (50 μg/ml) for 1 h at 55°C, phenol extracted, ethanol precipitated then re-suspended in Tris -ethylenediaminetetraacetic acid (EDTA). Negative controls of water only were incorporated into each batch of 22 extractions. Nested primers were selected from major outer membrane protein (MOMP) *omp 1* alignments. The outer (first round) primers used were APNOU (5′ AAT TCT CTG TAA ACA AAC CC 3′) and APNOL (5′ ATT AAG AAG CTC TGA GCA TA 3′), and the inner (second round) primers were APN1 (5′ AGC CTA ACA TGT ACA CTC TGA T 3′) and APN2 (5′ TGC CAA CAG ACG CTG GCG T 3′) generating a 487 base pair (bp) product detected by gel electrophoresis.

The PCR reaction buffers, AmpliTaq Gold. DNA polymerase and oligonucleotide primers were provided by Perkin Elmer Applied Biosystems (Norwalk, UK). A short (550 bp) cloned fragment of *Cp* genome DNA and two clinical samples known to be positive for Cp were used as positive controls. Negative controls were non-infected phosphate buffered saline (PBS). All positive samples were reconfirmed by repeat analysis and all DNA extractions, primary PCR amplifications and secondary PCR amplifications were undertaken in distinct areas, using dedicated pipettes and filtered tips.

Blood samples

Blood cytometric evaluation included a differential white cell count and eosinophil percentage as previously described (14).

Microimmunofluorescence

One serum sample from each subject was stored at −70°C before processing. Serum samples were

tested by microimmunofluorescence (MIF) for immunoglobulin (Ig)G, IgA and IgM antibodies to purified Cp elementary bodies (strain 2023, ATCC VR 1356) with a Chlamydia MIF Kit (Diamedix, Miami, FL, USA). The prevalence of Cp-specific IgG, IgA, and IgM antibodies was based on the presence of IgG at a titer of 1:32, IgA at a titer of 1:16, and IgM at a titer of 1:64. A positive reaction was seen as sharply defined apple-green fluorescent Cp elementary bodies in the cytoplasm of the infected cells. A negative reaction was seen as either red-counterstained cells or a hazy indistinct apple-green fluorescence that was indicative of anti-lipopolysaccharide reactivity.

Statistical analysis

Results were analyzed using the Statistical Package SPSS 9.0 for Windows (Chicago, IL, USA). Contingence tables were used for comparison between categorical variables, Fisher exact test was used whenever the expected frequency values were < 5; odds ratios (ORs) and 95% confidence intervals (CI) were used to express the likelihood of having both markers positive (PCR and serum IgG). p-values < 0.05 were considered to indicate statistical significance.

Results

The Questionnaire items yielded a study population comparable with previously reported samples in the prevalence of asthma, of other respiratory disorders and atopy (Table 1). PCR detected *Chlamydia* DNA was found in nasal aspirates from 20 of the 207 children tested (9.7%). IgG antibodies for *Chlamydia pneumoniae* were detected in 68 children (32.9%), IgA in 65 (31.4%), IgM in 23 (11.1%), IgG-IgA in 64 (30.9%) and IgG-IgM in 18 children (8.7%). Ninety-five percent of subjects with detectable IgG were also positive to IgA.

Table 1. Characteristics of the population of schoolchildren

No. subjects	207
Age (yr)	9.8 (s.d. 0.7)
Gender males (%)	45.9
Current or past asthma (%)	15.2
Exercise induced asthma (%)	4.9
Asthma in the past 12 months (%)	5.3
Eczema (%)	15.4
Persistent rhinitis (%)	13.1
Recurrent bronchitis/Chronic cough (%)	10.9
Atopy* (%)	25.6
Prick index (mm)†	25.6
Eosinophils >235 mm (%)‡	30.6

*At least one positive (≥3 mm wheal) skin prick test from seven local common antigen.
†Sum of all positive allergen (excluding *D. farinae*) reactions, in the 46 subjects with at least one positive allergen.
‡235 mm^3 is the 70th percentile of the eosinophil count in the studied population of school children.

Table 2. Prevalence of children with detectable or undetectable *Chlamydia pneumoniae* (Cp)-IgG antibodies among those with positive or negative nasal Cp-DNA detection

Cp-nasal DNA detection (PCR)	Serum Cp-IgG antibodies detection (MIF)		
	Positive	Negative	Total
Positive	15	5	20
Negative	53	134	187
Total	68	139	207

$p < 0.001$ by χ^2 test.

A strong association was found between the status of antigen carrier (PCR+) and the presence of detectable IgG in the serum ($p < 0.0001$; OR = 7.58; 95% CI 2.61–21.72) (Table 2). A similar association was found between the presence of detectable IgA and the status of nasal carrier of Cp antigens.

Analysis of the distribution of detectable IgG among the 14 classes in our study population showed that 34 of 68 IgG+ children (50% of those who tested positive) clustered in only four classes ($p < 0.001$). Similarly, nine of the 20 children with detectable Cp DNA (45%) belonged in three of the 14 classes ($p = 0.027$).

No significant relationship was found between a history of asthma, persistent respiratory symptoms, eczema, atopy and antigen detection in the nasal aspirate, or the presence of serum Cp antibodies (Table 3).

Discussion

In agreement with others, in this study conducted in an unselected population of Italian schoolchildren aged 9–10 yr, we found Cp DNA in 10% of the upper airways (nasal aspirates), and detectable Cp serum antibodies (IgG-IgA) in more than 30% of the children.

Contrary to several previous epidemiological reports (4), but in agreement with some others (17–20), we found no association between Cp (nasal DNA or antibody production) and the presence of asthma, chronic or recurrent respiratory symptoms, and atopy. The reported association between Chlamydia and asthma may therefore be too weak to be revealed in a study conducted on an unselected childhood population, inherently including few children with asthma.

The most intriguing finding of our study is the strong association between the status of Cp nasal

Table 3. Prevalence of children with nasal detectable *Chlamydia pneumoniae* (Cp) serum antibodies or DNA (PCR)

Children	Positive				Negative
	with Cp IgG	with Cp IgA	with Cp IgM	with Cp nasal DNA	
Total number [no. (%)] 207 (100)	68 (32.9)	65 (31.4)	23 (11.1)	20 (10.1)	131 (63.2)
Current or past Asthma (%)	13.4	15.6	8.7	15.0	16.7
Asthma in the past 12 months (%)	6.0	6.2	4.4	5.0	5.6
Exercise induced asthma (%)	2.9	4.7	4.5	5.0	5.6
Persistent rhinitis (%)	7.9	8.2	13.0	5.0	17.1
Recurrent bronchitis and chronic cough (%)	12.0	12.9	9.5	10.0	10.9
Eczema (%)	15.1	15.9	13.6	10.0	16.1
Atopy (%)	21.4	20.0	19.0	16.7	27.6
Prick index >7.7 mm	8.9	9.1	8.7	11.1	13.3
Eosinophils >235 mm^3	26.4	25.5	31.3	29.4	33.0

No significant association was found.

carrier and the presence of serum antibodies: it confirms a widely reported (but infrequently commented) finding. Many previous studies, report that detection of the organism (by culture or PCR) in the pharynx is accompanied by detectable serum antibodies (IgG or IgA against Cp) more frequently than expected by chance (21, 22). This DNA-antigen agreement in the same individual has been demonstrated in adults and in children (over the age of 5) and in healthy individuals and persons with acute respiratory infections or wheezing (23).

Although some have interpreted the finding of Cp antibodies in conjunction with positive PCR results as 'the response to an ongoing infection' (21) this cannot be the sole explanation. This Cp antigen-antibody association in the same individuals, suggests two possible mechanisms. First, that the micro-organism remains in the pharynx of infected individuals long enough for the host to mount an antibody response. Alternatively, those individuals who have been previously infected and responded with antibody production undergo frequent re-infections; hence they are unexpectedly frequently found to be carriers of Cp. Both these hypotheses imply a peculiar immune response to Cp in these individuals. It is self evident that the individuals who are not able to become readily free of the pathogen after an initial infection have some deficit in the immune response. Data are lacking on the frequency of this persistence at the epidemiological level but certainly the production of serum antibodies does not automatically follows Cp colonization in all individuals (19). For example even if Cp is commonly found in the upper airways of infants and children aged < 5 (24, 25, 26), serum Cp antibodies are only exceptionally found in the first years of life (27, 28). Moreover at any age males have higher prevalence than females (29, 30). Because of the long half-life of Cp serum antibodies it is surprising that no more than 50–60% of any adult population is found with detectable serum antibodies against Cp (31–33).

Taken together all these data suggests that the portion of the population carrying detectable Cp antibodies or who harbor Cp in their pharynx, and especially those with both these findings, have a peculiar immunologic behavior in occasion of the contact with this infectious agent.

In the life cycle of Cp, the cultivable extracellular elementary body attaches to and penetrates into the target cell, where it assumes the form of a metabolically active 'reticulate body' that can persist for long periods before transforming into an 'elementary body', which is then released from the cell by exocytosis or cell rupture (34). This 'parasitic' infection can be eradicated from the cell only by mean of a vigorous Th1 response (e.g., by an increase IFNγ); whereas a predominantly Th2 response favors a persistent infection (2, 35, 36). The role of CD4 lymphocytic helper cells has been well documented in CD8 knock-out mice (37): in these mice the lack of suppressor activity favors CD4 helper activity causing Cp to multiply excessively in infected animals. This overwhelming multiplication is accompanied by intense IL-4 production and reduced interferon-gamma production, a typical Th2 behavior. In CD8 knock-out mice the unopposed CD4 activity also favors tolerance to a chronic Cp infection that nevertheless leaves antibody production unchanged (32).

Given these epidemiological and laboratory observations, the proven positive association between markers of Cp infection and asthma, especially between Cp-PCR positive reactions and childhood asthma, comprehensively reviewed by Hahn (4), might not indicate that a previous

exposure or a chronic infection with Cp favors the development of asthma. It might instead reflect the known association of asthma with Th2-oriented lymphocytic activity. This Th2 prevalence could diminish the individual's ability to eradicate Cp and owing to prolonged or chronic antigen stimulation, increase their ability to produce antibodies against this microorganism. In this view, Cp should be considered simply as 'associated' with and not as a cause of asthma. The propensity to harbor or not to eliminate the infective agent and the predisposition to develop asthma could be two parallel consequences of a Th2-oriented cellular immunity.

An even more speculative question is whether these doubts and interpretations should be extended to the proven but weaker association of Cp with other chronic and degenerative conditions such as coronary or ischemic heart diseases (7, 8), or atherosclerosis and aortic aneurysms (6), cholesteatoma (9) or other clinical conditions such as sudden infant death syndrome (10).

References

1. Kuo CC, Jackson LA, Campbell LA, Grayston JT. *Chlamydia pneumoniae* (TWAR). Clin Microbiol Rev 1995: 8: 451–61.
2. Hammerschlag MR, Chirgwin K, Roblin PM, et al. Persistent infection with *Chlamydia pneumoniae* following acute respiratory illness. Clin Infect Dis 1992: 14: 178–82.
3. Falck G, Heyman L, Gnarpe J, Gnarpe H. *Chlamydia pneumoniae* and chronic pharyngitis. Scand J Infect Dis 1995: 27: 179–82.
4. Hahn MS. *Chlamydia pneumoniae*, asthma, and COPD: it is the evidence? Ann Allerg 1999: 23: 271–91.
5. Saikku P, Leinonen M, Tenkanen L, et al. Chronic *Chlamydia pneumoniae* infection as a risk factor for coronary heart disease in the Helsinki heart study. Ann Intern Med 1992: 116: 273–8.
6. Gaydos C.A. Growth in vascular cells and cytokine production by *Chlamydia pneumoniae*. J Infect Dis 2000: 181(Suppl. 3): S473–8.
7. Thom DH, Grayston JT, Siscovick DS, Wang SP, Weiss NS, Daling JR. Association or prior infection with *Chlamydia pneumoniae* and angiographically demonstrated coronary artery disease. JAMA 1992: 268: 68–72.
8. Rosenfeld ME, Blessing E, Lin TM, Moazec TC, Campbell LA, Kuo C. *Chlamydia*, inflammation, and atherogenesis. J Infect Dis 2000: 181(Suppl. 3): S492–7.
9. Ronchetti F, Ronchetti R, Guglielmi F, et al. Detection of *Chlamydia pneumoniae* in cholesteatoma tissue: any pathogenetic role? Otol Neurotol 2003: 24: 353–7.
10. Lundemose JB, Lundemose GA, Gregersen M, et al. *Chlamydia* and sudden infant death syndrome. A study of 166 SIDS and 30 control cases. Int J Legal Med 1990: 104: 3–7.
11. Falck G, Gnarpe J, Gnarpe H. Prevalence of *Chlamydia pneumoniae* in healthy children and in children with respiratory tract infections. Pediatr Infect Dis 1997: 16: 549–54.
12. Ferris BG. Epidemiology Standardization Project. Recommended respiratory disease questionnaires for use with adults and children in epidemiological research. Am Rev Resp Dis 1978: 118: 7–53.
13. Barreto M, Villa MP, Martella S, et al. Exhaled nitric oxide in asthmatic and no asthmatic children: influence of the type of allergen sensitization and exposure to tobacco smoke. Pediatr Allergy Immunol 2001: 12: 247–56.
14. Ronchetti R, Villa MP, Martella S, et al. Nasal cellularity in 183 unselected schoolchildren aged 9–11 years. Pediatrics 2002: 110: 1137–42.
15. Ronchetti R, Bonci E, Cutrera R, et al. Enhanced allergic sensitisation related to parental smoking. Arch Dis Child 1992: 67: 496–500.
16. Johnston SL, Sanderson G, Pattemore PK, et al. Use of polymerase chain reaction for diagnosis of picornavirus infection in subjects with and without respiratory symptoms. J Clin Microbiol 1993: 31: 11–7.
17. Mills GD, Lindeman JA, Fawcett JP, et al. *Chlamydia pneumoniae* serological status is not associated with asthma in children or young adults. Int J Epidemiol 2000: 9: 280–4.
18. Lieberman D, Lieberman D, Printz S, et al. Atypical pathogen infection in adults with acute axacerbation of bronchial asthma. Am J Respir Crit Care Med 2003: 167: 406–10.
19. Foschino Barbaro MP, Resta O, Aliani M, et al. Seroprevalence of chronic *Chlamydia pneumoniae* infection in patients affected by chronic stable asthma. Clin Microbiol Infect 2002: 8: 358–62.
20. Schmidt SM, Muller CE, Bruns R, et al. Bronchial *Chlamydia pneumoniae* infection, markers of allergic inflammation and lung function in children. Pediatr Allergy Immunol 2001: 12: 257–65.
21. Hyman CL, Roblin PM, Graydos CA, Quinn TC, Schachter J, Hammerschlag MR. Prevalence of asymptomatic nasopharyngeal carriage of *Chlamydia pneumoniae* in subjectively healthy adults: assessment by polymerase chain reaction-enzyme immunoassay and culture. Clin Infect Dis 1995: 20: 1174–8.
22. Ferrari M, Poli A, Olivieri M, et al. Seroprevalence of *Chlamydia pneumoniae* antibodies in a young adult population sample living in Verona. European Community Respiratory Health Survey (ECRHS) Verona. Infection 2000: 28: 38–41.
23. Gnarpe J, Gnarpe H, Sundelöf B. Endemic prevalence of *Chlamydia pneumoniae* in subjectively healthy persons. Scand J Infect Dis 1991: 23: 387–8.
24. Falck J, Gnarpe J, Gnarpe H. Persistent *Chlamydia pneumoniae* infection in a Swedish family. Scand J Infect Dis 1997: 28: 271–3.
25. Normann E, Gnarpe J, Gnarpe H, Wettegren B. *Chlamydia pneumoniae* in children with acute respiratory tract infection. Acta Pediatr 1998: 87: 23–7.
26. Block SL, Hammerschlag MR, Hedrick J, et al. *Chlamydia pneumoniae* in acute otitis media. Pediatr Infect Dis J 1997: 16: 858–62.
27. Saikku P, Ruutu P, Leinonen M, Panelius J, Tupasi TE, Grayston JT. Acute lower respiratory tract infection associated with Chlamydial TWAR antibody in Filipino children. J Infect Dis 1988: 158: 1095–7.
28. Wang SP, Grayston JT. Population prevalence antibody to *Chlamydia pneumoniae* strain TWAR. In:

Bowie WR, Caldwell HD, Jones RP, et al. eds. Chlamydial infections. Cambridge: Cambridge University Press, 1990: 402–5.
29. Blasi F, Cosentini R, Schoeller MC, Lupo A, Allegra L. *Chlamydia Pneumoniae* seroprevalence in immunocompetent and immunocompromised populations in Milan. Thorax 1993: 48: 1261–3.
30. Karvonen M, Tuomilehto J, Pitkaniemi J, Saikku P. The epidemic cycle of *Chlamydia pneumoniae* infection in eastern Finland, 1972–1987. Epidemiol Infect 1993: 110: 349–60.
31. Aldous MB, Grayston JT, Wang SP, Foy H. Seroepidemiology of *Chlamydia pneumoniae* TWAR infection in Seattle families, 1966–1970. J Infect Dis 1992: 166: 646–9.
32. Einarsson S, Sigurdsson HK, Magnusdottir SD, Erlensdottir H, Briem H, Gudmunsson S. Age specific prevalence of antibodies against *Chlamydia pneumoniae* in Ireland. Scand J Infect Dis 1994: 26: 393–7.
33. Saikku P. The epidemiology and significance of *Chlamydia pneumoniae*. J Infect Dis 1992: 25 (Suppl. 1): 27–34.
34. Cook PJ, Honeybourne D. *Chlamydia pneumoniae*. J Antimicrob Chemother 1994: 34: 859–73.
35. Holland MJ, Bailey RL, Conway DJ, et al. T helper type-1 (Th1/Th2 profiles of peripheral blood mononuclear cells (PMBC); responses to antigens of *Chlamydia trachomatis* in subjects with severe trachomatous scarring. Clin Exp Immunol 1996: 105: 429–35.
36. Von Hertzen LC. Role of persistent infection in the control and severity of asthma: focus on *Chlamydia pneumoniae*. Eur Respir J 2002: 19: 546–56.
37. Rottemberg ME, Gigliotti Rothfuchs A, Gigliotti D, et al. Role of innate and adaptive immunity in the outcome of primary infection with *Chlamydia pneumoniae*, as analysed in genetically modified mice. J Immunol 1999: 162: 2829–36.