Eur Respir J 2000; 16: 108–111
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European Respiratory Journal
ISSN 0903-1936

Chlamydia pneumoniae infection in adults with chronic cough compared with healthy blood donors

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Chlamydia pneumoniae *infection in adults with chronic cough compared with healthy blood donors. N.H. Birkebæk, J.S. Jensen, T. Seefeldt, J. Degn, B. Huniche, P.L. Andersen, L. Østergaard. ©ERS Journals Ltd 2000.*

ABSTRACT: In a small uncontrolled study, persistent cough has recently been found to be associated with serological evidence of acute *Chlamydia pneumoniae* infection. In order to assess whether *C. pneumoniae* plays a role in chronic cough, the prevalence of *C. pneumoniae* infection in 201 adult patients with chronic cough was compared with the prevalence in 106 healthy blood donors without respiratory tract symptoms in the preceding 3 months. A microimmunofluorescence antibody test was used to determine *C. pneumoniae* antibodies in the immunoglobulin (Ig)M, IgG and IgA fractions. Further, nasopharyngeal aspirates from the 201 patients were examined for *C. pneumoniae* deoxyribonucleic acid by polymerase chain reaction (PCR).

As judged by serology, nine patients (4%) and one control (1%) had acute *C. pneumoniae* infection, and 92 patients (46%) and 42 controls (40%) had previous or chronic *C. pneumoniae* infection. Of the nine patients with acute infection, three were *C. pneumoniae* PCR positive, and they all had an IgM antibody titre response. The remaining six patients had either an IgG antibody titre of ≥512 (five patients) or an IgA antibody titre of ≥512 (one patient). None of these six patients had detectable IgM antibodies.

The mean cough period for the five IgG positive patients (10.8 weeks) was significantly longer than the mean cough period for the remaining patient population (6.4 weeks; p=0.004).

It is concluded that *Chlamydia pneumoniae* infection was not statistically significantly more prevalent in patients with chronic cough than in healthy blood donors, and that *Chlamydia pneumoniae* appears to have a minor role in patients with chronic cough. Direct detection of *Chlamydia pneumoniae* by polymerase chain reaction on nasopharyngeal aspirates is highly correlated with detectable immunoglobulin M antibodies, but in the late stages of prolonged cough serological testing of immunoglobulin G and immunoglobulin A may be more beneficial for obtaining a microbiological diagnosis.

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Keywords: Chronic cough
Chlamydia pneumoniae
healthy blood donors
polymerase chain reaction
serology

Received: June 23 1999
Accepted after revision March 13 2000

This study was supported by a grant from the Danish Lung Association, and by a grant from "Klinisk Institut", Odense University, Denmark.

Chlamydia pneumoniae has been associated with respiratory tract infections in children and adults [1–8]. Recently, it was shown, by use of serological methods, that 20% of an American adult population with persistent cough (2–12 weeks) had evidence of acute *C. pneumoniae* infection, but no healthy control population was included for comparison [9]. Among healthy adults [10] and healthy children [11] acute *C. pneumoniae* infection has been shown in 19% and 23%, respectively. It is therefore unresolved whether *C. pneumoniae* plays a role in chronic cough or whether it is just a commensal. In order to clarify this, the authors evaluated an adult population with chronic cough and a group of healthy blood donors for serological evidence of acute and chronic *C. pneumoniae* infection. It was also investigated whether *C. pneumoniae* could be detected by polymerase chain reaction (PCR) in nasopharyngeal aspirates in the patients with chronic cough.

Material and methods

Subjects and sampling

At the Dept of Pulmonary Medicine at Aarhus University Hospital, Denmark, 247 adult patients referred for prolonged cough from November 1995 to May 1997 were evaluated for the study. The inclusion criteria were age >15 yrs, a cough period of 2–12 weeks (which has previously been used as the expected time frame of a prolonged *C. pneumoniae* cough [9]) a normal chest radiograph, normal spirometry, and absence of chronic cardiopulmonary disease. Two-hundred-and-one patients fulfilled the inclusion criteria and were enrolled into the study. Forty-six patients did not participate for the following reasons: cough for >12 weeks in 15 patients, abnormal spirometry test in 19 patients, pneumonia in four patients, sarcoidosis in one patient, cardiomegaly in one patient; and six patients refused to participate.

The control population comprised 106 healthy blood donors (73 males and 33 females) without respiratory tract symptoms within the previous 12 weeks. Sera from the control population were obtained during the same period of time as the patients.

A medical history including information about age, sex, smoking habits, and cough was obtained for each patient. A chest radiograph (anterior-posterior and lateral projection) and spirometry were performed. From all patients a nasopharyngeal aspirate and a baseline serum sample were obtained. Four weeks later a follow-up serum sample was obtained from 188 patients. A baseline and a follow-up serum sample were obtained from the blood donors. Serum was stored at -20°C until examination, while nasopharyngeal aspirates were stored at -80°C until examination.

Chlamydia pneumoniae *polymerase chain reaction*

Deoxyribonucleic acid (DNA) from 100 µL of the nasopharyngeal aspirate was released by thorough vortexing with 300 µL of a 20% weight/volume Chelex 100 slurry (143-2832; Bio Rad Lab, Richmond, CA, USA) [12], followed by boiling of the suspension for 10 min; then centrifuged at 20,000×*g* for 10 min.

Ten microlitres of the supernatant was subjected to a PCR using primer pair 1A/1B [13] and corresponding mixed primers matching the 16S ribosomal DNA sequence of other Chlamydia species. A "touchdown" procedure [14] was used with a 1°C decrement of the annealing temperature for the first 10 cycles. A total of 50 cycles were performed. Amplicons were visualized by staining with ethidium bromide after agarose gel electrophoresis. Amplified material was labelled during PCR with digoxigenin-11-deoxyuridine triphosphate (dUTP) (Boehringer-Mannheim, Germany) at a molar ratio to unlabelled dUTP of 1:312. In order to lower the detection limit and to differentiate between the three chlamydial species, a liquid hybridization assay using biotinylated species specific probes was performed. This assay was capable of detecting <0.2 inclusion forming units.

An internal processing control consisting of a 618 base pairs phage lambda DNA flanked by the PCR primers was used in order to detect inhibition of the PCR. If inhibitory substances could be shown another PCR with 5 µL of the supernatant was performed.

Positive results were confirmed by a second nested PCR amplifying a part of the *C. pneumoniae* major outer membrane protein gene [4].

Mycoplasma pneumoniae *polymerase chain reaction*

Twenty-five microlitres of the pretreated sample was tested with primers P1-178/P1-331 deduced from the *Mycoplasma pneumoniae* P1 adhesin gene in a 40 cycle PCR. Amplicons were detected by agarose gel electrophoresis. Assay detection limit was 5–10 genome copies [15].

Chlamydia pneumoniae *serology*

Immunoglobulin (Ig)M, IgA and IgG antibodies against *C. pneumoniae* were detected by microimmunofluorescence antibody test (MIF) using TWAR antigen (Washington Research Foundation, Seattle, WA, USA). Incubation time was 30 min at 37°C at each incubation step. Sera with IgM against *C. pneumoniae* were retested after absorption with goat anti-human IgG antibody reagent (Gull SORB; Gull Laboratories, Salt Lake City, UT, USA), in order to exclude false positive reactions [16], and all sera were absorbed by Gull SORB before testing for IgA antibodies [17]. A positive and a negative control were included in each setup. Samples were blinded before serological testing. Acute and follow-up sera from each patient were analysed in parallel in the same experiment.

Conventionally, serological evidence of acute infection has been defined as a MIF IgM titre of ≥16, a MIF IgG titre ≥512 or a four-fold increase in IgG titre [16]. As proposed by EKMAN *et al.* [18] a MIF IgA titre ≥512 was also considered as evidence of acute infection. There are no firm criteria for serological evidence of previous or persistent infection, thus an IgG titre ≥64 and <512, or an IgA titre ≥16 and <512 were considered indicative of previous or persistent infection [19].

Mycoplasma pneumoniae *serology*

Complement fixation test was used to detect antibodies to *M. pneumoniae* [20]. A four-fold titre rise or a titre >256 was considered as evidence of an acute infection.

Statistics

For data analysis the statistical package for the social sciences (SPSS) version 6.0 [21] was used. For dichotomous variables the Chi-squared test was used, and for continuous variables an unpaired t-test. A p-value <0.05 was considered statistically significant.

Ethics

The study was approved by the ethics committee of the county. Before entering the study written informed consent was obtained from each patient.

Results

After stratification according to sex, the 201 patients and 106 healthy blood donors were comparable with regard to age and smoking habits (table 1). The patient and control groups had a different sex ratio, but no difference in antibody titres according to sex could be shown (table 1). Convalescent serum was not obtained from 13 patients. No difference between these patients and the other patients regarding history could be found. One of the 13 patients had a positive *C. pneumoniae* IgM antibody titre, the remaining 12 patients had a negative IgM and IgA antibody titre, and an IgG antibody titre of ≤128. Nine patients (five males and four females) had acute infection (table 2). Of these nine patients, three had an IgM antibody titre of 512 and an IgG and IgA antibody titre <512, whereas five had IgG antibody titres ≥512 and one had an IgA antibody titre of 512. No patients had a four-fold change in any Ig class. Absorption with goat antihuman IgG antibody did not change the IgM titre of the IgM positive patients. One male healthy blood donor had an

Table 1. – Demographic and *Chlamydia pneumoniae* serological data of the 201 patients and the 106 healthy blood donors stratified according to sex

Characteristics	Patients		Control		p-value
	Male	Female	Male	Female	
Number	69	132	73	33	
Age yrs*	41±13	42±15	41±10	39±10	NS
Smoker#	25 (36)	42 (32)	22 (30)	8 (24)	NS
MIF IgM ≥16 or MIF IgG ≥512 or MIF IgA ≥512#	5 (7)	4 (3)	1 (1)	0	NS
MIF IgG ≥64 and <512 or MIF IgA ≥16 and <512#	29 (42)	63 (48)	30 (41)	12 (36)	NS

*: mean±SD; #: absolute number with percentage in parentheses. NS: nonsignificant.

IgG antibody titre of 512 (table 2). Ninety-two (46%) of the patients and 42 (40%) of the healthy blood donors had an IgG antibody titre ≥64 and <512 or an IgA antibody titre ≥16 and <512 (table 1; not significant).

The results of *C. pneumoniae* PCR, baseline serological testing and coughing time for patients with serological evidence of an acute *C. pneumoniae* infection are shown in table 2. As can be seen in the table, three patients had a positive *C. pneumoniae* PCR (1.5%) and these patients all had a positive IgM titre. No patient was positive by PCR and had a negative IgM titre, and no patient had a positive IgM titre and a negative PCR test. The six patients that had IgM titres <16 and high IgG or IgA titres were all PCR negative. The five patients with high IgG titres had been coughing for ≥8 weeks (mean 10.8 weeks), compared with a mean coughing time for the remaining patient group of 6.4 weeks (p=0.004).

No patient was *M. pneumoniae* PCR or serology positive.

Substances inhibitory to PCR could be shown in 48 of the nasopharyngeal aspirates. However, when the DNA samples were diluted two-fold, the internal control was amplified in all samples indicating that the inhibitory substances were diluted to a concentration which did not inhibit the PCR reaction. However, no further samples were *C. pneumoniae* PCR positive.

Table 2. – Microbiological data and coughing time of the nine patients with serological evidence of acute *Chlamydia pneumoniae* infection

Case no.	Sex	Age yrs	PCR	IgM titre I	IgA titre I	IgG titre I	Coughing time weeks
1	M	43	-	<16	128	1024	12
2	F	30	+	512	64	64	2
3	F	57	-	<16	128	1024	12
4	M	50	-	<16	128	512	12
5	F	58	-	<16	<16	512	10
6	F	26	+	512	128	128	>2 and <12
7	M	40	+	512	256	64	3
8	M	49	-	<16	64	512	8
9	M	52	-	<16	512	256	6

PCR: polymerase chain reaction; Ig: immunoglobulin; M: male; F: female.

Discussion

High antibody titres indicating acute *C. pneumoniae* infection have been found in patients with chronic cough [9], but *C. pneumoniae* in throat swabs as well as high *C. pneumoniae* antibody titres have also been found in healthy adults [10, 22]. Furthermore, sex, age and smoking habits might influence *C. pneumoniae* serology [23–25]. Therefore, in order to study the prevalence of *C. pneumoniae* in patients with chronic cough, the authors included a control population without respiratory symptoms in the previous 3 months. The patients and control subjects were comparable with regard to age and smoking habits, and no differences in antibody titres according to sex were found. Patient and control samples were collected in the same time frame.

Using MIF serology, evidence of acute *C. pneumoniae* infection was found, as indicated by high IgM, IgG or IgA antibody titres in 4% of the patient population and in 1% of the healthy controls. In an American study of patients with persistent cough, 20% had evidence of acute infection, using the same IgM and IgG serological criteria for *C. pneumoniae* infections as was used in the current study [9]. Although the present study included a high IgA antibody titre as an indication of acute infection [18], the low prevalence of *C. pneumoniae* in this patient population compared with the American study, might be explained by periodicity of *C. pneumoniae* infection [11] as well as by the exclusion of patients with obstructive lung disease, who may have been at a higher risk of *C. pneumoniae* infection [6, 26]. In another study of *C. pneumoniae* antibody titres in healthy adults, 19% fulfilled the serological criteria of acute infection compared with only 1% in the current study [10]. The study by HYMAN *et al.* [10] was performed in healthcare workers, who may have an increased risk of *C. pneumoniae* exposure, which may explain the high prevalence of *C. pneumoniae* in this population.

Five patients with an IgG antibody titre indicating acute infection [16] had been coughing significantly longer than the remaining patient population, and all five were IgM antibody negative and PCR negative. The findings indicate that in these five patients, the IgM antibody response and the *C. pneumoniae* DNA may have disappeared by the time the patient seeks a physician for testing; or the patients might have had a reinfection, during which IgM antibody responses are usually absent [16]. Serology may thus be more reliable than PCR in detecting *C. pneumoniae* infection in late stages of chronic cough. On the other hand, the idea that the presence of a high IgG titres without a significant change over a 4-week period, may lack specificity for diagnosing recent or current infection cannot be excluded.

Previous or persistent infection with *C. pneumoniae* did not correlate with chronic cough since this was found in 46% of patients and in 40% of healthy blood donors. Similar or even higher prevalences have been found in other studies [7, 10, 27, 28]. This indicates that over time the same fraction of people are infected by *C. pneumoniae*, whether or not they have a chronic cough.

The sampling technique for respiratory specimens for *C. pneumoniae* PCR, the preparation of the sample for PCR and the PCR system itself might influence the sensitivity of the test [2, 29, 30]. However, agreement about the optimal sampling method has not yet been reached [2, 29, 31].

The current study used Chelex for releasing DNA from nasopharyngeal aspirate specimens for *C. pneumoniae* PCR. The Chelex method is rapid and gives a relatively low risk of contamination, and has been found to be as effective as the "gold standard" for DNA extraction, *i.e.* phenol/chloroform extraction [4]. The PCR system was able to detect <0.2 inclusion forming units. Thus the sparse detection rate of *C. pneumoniae* by PCR in the study seems reliable, and cannot be explained by insensitivity of the method. Furthermore, the complete agreement with the detection of *C. pneumoniae* IgM indicates its value in the detection of acute infection.

It is concluded that *Chlamydia pneumoniae* infection was not statistically significantly more prevalent in patients with chronic cough than in healthy blood donors, and that *Chlamydia pneumoniae* cannot therefore be considered as a major pathogen in patients with chronic cough. Direct detection of *Chlamydia pneumoniae* by polymerase chain reaction on nasopharyngeal aspirates may be less useful than serology in the late stages of chronic cough. However, a lack of diagnostic specificity of the currently used serological criteria cannot be excluded.

Acknowledgements. The authors thank J. Guldberg, B. Dohn and L. Berthelsen for their excellent technical assistance.

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