Detection of *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells of blood donors in the north-east of Italy

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Abstract Recent studies have implicated *Chlamydia pneumoniae* (now *Chlamydia pneumoniae*) in the pathogenesis of atherosclerosis and demonstrated its presence within human peripheral blood mononuclear cells (PBMCs). In this study the presence of *C. pneumoniae* DNA was assessed, using nested PCR, in PBMCs from 169 active blood donors as a function of age, of specific antibodies and C-reactive protein. The results obtained demonstrated a high degree of global positivity (46.15%), which was higher in females (52%) than in males (43.7%). Seroepidemiological studies showed a high percentage of positivity both in subjects positive by PCR (65.91%) and negative by PCR (71.74%). The clinical implication of such finding are under study.

Keywords *Chlamydia pneumoniae* · Polymerase chain reaction · Blood donors · Peripheral blood mononuclear cells · Antibodies

Introduction

In the last few years a number of studies have tried to link atherosclerosis with viral or bacterial infections. More specifically, three agents have been claimed as possible predisposing factors for atherosclerosis by seroepidemiological studies: *Chlamydia pneumoniae*, *Helicobacter pylori* and cytomegalovirus [9]. Among these, the most convincing evidence of a causal association with atherosclerosis (based on seroepidemiological, pathological, animal model, and in vitro studies) [23, 24, 25, 28] has been provided for *C. pneumoniae*, a common human bacterial pathogen that causes community acquired pneumonias, bronchitis, and sinusitis [15]. *Chlamydia pneumoniae* (now *Chlamydia pneumoniae*) [11] is a distinct species within the genus *Chlamydia*, which includes a group of obligately intracellular bacteria that have a unique developmental cycle and pathogenicity. They are parasites of humans and a wide variety of animals. Seroepidemiological studies have shown that more than 60% of adults are infected with *C. pneumoniae* during their lifetime [14]. Saikku et al. [28] reported a close association between high level of *C. pneumoniae*-specific IgA antibodies and an increased risk for myocardial infarction, although this data is controversial [10, 19]. The detection of *C. pneumoniae*-specific antibodies may be due to a previous or an ongoing *C. pneumoniae* infection. Therefore, to identify *C. pneumoniae* carriers and to measure the effect of antibiotic therapy, a sensitive and specific method, such as the nested PCR (nPCR)-based detection of *C. pneumoniae* DNA may prove useful [7]. Interestingly, with this approach, Boman et al. [6] showed that the presence of *C. pneumoniae* DNA in circulating peripheral blood mononuclear cells (PBMCs) is common in seropositive coronary heart disease patients. Blasi et al. [4] demonstrated that the rate of *C. pneumoniae* detection DNA in artery specimens and
PBMCs was similar. Thus, PCR performed on PBMCs may be a good marker for the identification of subjects carrying *C. pneumoniae*. The accumulating evidence pointing to an involvement of *C. pneumoniae* in the pathogenesis of atheromas (ATS) plaques contrasts with the lack of information on the mechanisms taking place during plaque formation and on the precise role of *C. pneumoniae*. A recent report suggests a putative role for chlamydia antigens, not related to the presence of viable bacteria [22, 30]. Since the demonstration of *C. pneumoniae* on PBMCs has been confirmed by a large series of studies and since asymptomatic individual could also present circulating *C. pneumoniae* DNA on their PBMCs, the possibility that *C. pneumoniae* DNA could be transferred to other subjects by blood transfusion should be taken into consideration.

The purpose of the present study was to investigate the presence of *C. pneumoniae* DNA in the peripheral blood from regularly active blood donors at the time of their donation, and correlate it with donor’s age and timing of blood donation. A correlation was also made with the presence of specific antibodies.

**Methods**

Whole venous blood (8 ml) was collected in EDTA-treated tubes from 169 blood donors (119 males and 50 females). For serology, 5 ml of venous blood were collected in sterile tubes. All samples were collected between February and October 2000 in north-eastern Italy, and there was no evidence of an ongoing epidemic of *C. pneumoniae* in the community; recent upper respiratory tract infection and use of antibiotics 2 weeks before and during the studies were considered exclusion criteria. Informed consent was obtained from all subjects prior to admission to the study. Blood samples for nPCR were processed in accordance with a method described by Condos et al. [8]; 8 ml of whole blood were collected from each patient in Vacutainer CPT (Becton Dickinson, Franklin Lakes, New Jersey), for isolation of PBMCs. A red blood cell lysis buffer (ammonium chloride solution: 0.1 M NH₄Cl, 1 M KHCO₃, 0.1 M Na₂EDTA.2H₂O) [27] was then added to the blood sample to a final volume of 50 ml, gently mixed and left 10 min at room temperature to allow the lysis of red blood cells. The tube was then centrifuged at 1,500 g for 10 min at room temperature, the supernatant was discarded and the pellet resuspended in phosphate-buffered saline (PBS), transferred to an eppendorf tube and centrifuged for 10 min at 1,500 g. The supernatant was discarded again and the pellet transferred at −70°C until DNA extraction was performed.

**PCR analysis**

The specimens were thawed on ice, resuspended in 40 μl of bidistilled water and digested overnight in 10 mM TRIS-HCl pH 8.3 and 50 mM KCl with proteinase K at a final concentration of 0.5 g/l and 0.6% (v/v) Nonidet P-40 at 55°C. The mixture was then boiled for 10 min and extracted twice with phenol-chloroform and once with chloroform only. DNA was precipitated with 0.1 vol 3 M sodium acetate and 2 vol ice-cold absolute ethanol, then pelleted, air-dried in a laminar flow environment and resuspended in sterile bidistilled water. To avoid the risk of contamination, DNA extraction, PCR amplification, and electrophoresis were performed in separate rooms. A negative and a positive control were run in each assay. The negative control contained all of the PCR reagent and sterile distilled water. Amplification of a fragment of the β-globin gene was used as internal control [26]. Touchdown nPCR was performed essentially as previously described [25]. The gene coding for the major outer membrane protein (ompA) was amplified. A nested protocol with the outer pair of primers (CP1, 5′-TTCAGCTTGCTGTAGG-3′; CP2, 5′-GCGATCCAAATGTATGAAGC-3′), common to both the ompA gene of *C. pneumoniae* and *C. psittaci*, and inner pair of primers (CP3, 5′-TTATATGTTGTTACAATA-3′; CPD, 5′-ATCTACGGCATATGATATATG-3′) specific for a variable domain of the ompA gene of *C. pneumoniae* was used [29]. The amplification product obtained with the outer set of primers was a 333-base pair (bp) fragment, while the second amplification yielded a 207-bp amplification fragment. The PCR reaction mix for the first amplification was as follows: 5 μl template DNA, 67 mM TRIS-HCl pH 8.8, 16 mM (NH₄)₂SO₄, 0.01% Tween 20, 25 pmol of each CP1 and CP2, 200 μM each of dNTPs, 1.25 U Taq polymerase (Eurobio, France) and 60 mM MgCl₂ in a final volume of 50 μl. Amplification was performed with a Perkin Elmer 2400 thermal cycler using a “Touchdown PCR” [29] technique in which the annealing temperature was lowered 1°C every two cycles from 65°C to 55°C. Then 20 more cycles were performed with annealing at 55°C; the denaturation steps were all performed at 94°C and the extensions at 72°C, the holding time at each temperature was 1 min. Of the first amplification 2 μl was used as a template for the second amplification. The PCR reaction mix was: 67 mM TRIS-HCl pH 8.8, 16 mM (NH₄)₂SO₄, 0.01% Tween 20, 3.0 mM MgCl₂, 200 μM each of each dNTPs, 50 pmol of each PCR primers in a final volume of 50 μl. The second round of PCR consisted of 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min; a final extension step of 7 min at 72°C was performed. The amplification products were visualized in 3% NuSieve 3:1 agarose (FMC Bioproducts, USA) with ethidium bromide staining by standard techniques. All samples were tested at least twice before reporting. Samples which gave discrepant results when retested were not considered in this study. Identity of the amplified product with the published sequence of the ompA fragment was performed by restriction analysis. The inner primers was cut with *Sma*I and *Alu*I (Boehringer Mannheim) and run on a 12% polyacrylamide gel. Purified *C. pneumoniae* DNA from strain IOL-207, obtained from the laboratory of the S. Maria degli Angeli Hospital (Pordenone, Italy), was used as positive control. Identity of this strain with type strain TWAR ATCC VR 1310 was demonstrated by 16 S sequencing and alignment [13, 16].

**Serology analysis**

*C. pneumoniae* serology was performed in 90 subjects. We relied on a commercially available microimmunofluorescence (MIF) technique for IgG, IgA and IgM (EuroImmun. Gross-Gronau, Germany) described in detail elsewhere [3, 31].

C-reactive protein (CRP) presence was assayed on all blood donors using an automated nephelometric immunoassay using a Beckman Array multistest immunoassay system. The detection range for CRP by this method is between 0.4 and 12 mg/100 ml. The test is standardized by the International Federation of Clinical Chemistry (IFCC) International Reference Preparation for Plasma Proteins.

**Statistical analysis**

The difference between males and females with respect to PCR detection of *C. pneumoniae* DNA was assessed by χ² test. The effect of age as a possible confounding factor was adjusted by stratification. The common risk was calculated with Cochran-Mantel-Haenszel statistics [21]. Moreover, the prevalence of a PCR-positive finding and the prevalence of a positive antibody test for the same microorganism across age strata were subjected to analysis according to linear model as described by Fleiss [12].