REAL-TIME POLYMERASE CHAIN REACTION AND LASER CAPTURE MICRODISSECTION: AN EFFICIENT COMBINATION TOOL FOR *CHLAMYDOPHILA PNEUMONIAE* DNA QUANTIFICATION AND LOCALIZATION OF INFECTION IN ATHEROSCLEROTIC LESIONS

A. CIERVO, F. MANCINI, P. SALE[1], A. RUSSO[2] and A. CASSONE

Department of Infectious, Parasitic and Immuno-mediated Diseases, Istituto Superiore di Sanità, Rome; [1]IRCCS San Raffaele Pisana, Roma; [2]IRCCS Regina Elena, Rome, Italy

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***Chlamydophila pneumoniae* has been implicated in atherosclerosis, but the role of this obligate intracellular pathogen in the development of the above pathology is still unclear. In particular, its presence and quantitative distribution within lesional areas has not yet been defined. We studied 18 carotid biopsies obtained from patients undergoing endoartherectomy. By laser microdissection (LCM), two different sites (intra-plaque and plaque-adjacent areas) were taken from each lesion, and the presence and quantity of the pathogen DNA were determined by real-time polymerase chain reaction (Real-time PCR). A total of 8 plaques, exclusively from patients with unstable angina, were positive in real-time PCR. The bacterial DNA was detected in both lesional areas of 3 plaques which contained the highest number of DNA copies (1,900 to 2,200 copy numbers), while *C. pneumoniae* DNA was detected only in the intra-plaque area of the other 5 positive (500 to 1,600 copy numbers). No *C. pneumoniae* DNA was found in the other 10 plaques of which 6 were from patients with unstable angina and 4 from stable angina patients. No DNA from *Helicobacter pylori* or Cytomegalovirus was found in any plaque. This is the first report where both the target lesion and an adjacent reference site were evaluated for the presence of *C. pneumoniae* DNA by the combination of LCM and Real-time PCR assays. The integration of these two methodologies offer an excellent tool for *in situ* studies and may help to elucidate the putative role of *C. pneumoniae* in atherosclerosis.**

Laser capture microdissection (LCM) has been proposed as potential application in human pathology to isolate material of interest for molecular evaluation, and it has been successfully applied in cancer and cardiovascular research (1). When combined with molecular tools, such as PCR or 2D-electrophoresis, LCM allows to assess whether a given genome or a proteomic profile is present in certain cells or parts of a tissue and not in any other close tissue areas. Concerning atherosclerosis, overexpression of human macrophage genes was detected in atherosclerotic and macrophage-rich areas (2), while Martinet et al. were able to detect caspase-2 overexpression with anti-apoptotic effects (3).

For some time we have been studying the aetiological or pathogenic association of *Chlamydophila pneumoniae* with cardiovascular

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Mailing address: Dr Alessandra Ciervo, PhD
Department of Infectious, Parasitic and Immuno-mediated Diseases,
Istituto Superiore di Sanità,
Viale Regina Elena 299, 00161 Rome, Italy
Tel: ++39 06 49903127 Fax: ++39 06 49387183
e-mail: alessandra.ciervo@iss.it

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disease, and have recently documented the sparse presence of *C. pneumoniae* genome in carotid plaques by real-time polymerase chain reaction (Real-time PCR) (4). For the above association, detection of *C. pneumoniae* in atheromatous plaques has become a critical, yet still unresolved, issue. Laboratory methods for detecting individuals infected with *C. pneumoniae* have until now mainly relied on serological analyses, microimmunofluorescence (MIF) in particular, since culturing the organism from clinical samples is rather difficult and associated with low yield. *C. pneumoniae* detection by polymerase chain reaction (PCR)-based methods have been shown to be sensitive and specific but are not yet standardized or widely available (5). Moreover, when applied to bulk tissue the technique does not allow to establish in which cell type the amplified product has been generated. Overall, *C. pneumoniae* DNA detection in atheromatous lesions by PCR assays remains problematic for a microorganism presumed to be present at low levels in the plaque.

For all these reasons, coupling LCM with sensitive Real-time PCR can prove of remarkable usefulness in tracking *C. pneumoniae* DNA localization and quantification in the atherosclerotic plaques. We present here the results of the application of Real-time PCR and LCM to 18 biopsies from carotid endoatherectomy for the presence and number of *C. pneumoniae* genomes .

MATERIALS AND METHODS

Patient and samples

A total of 18 biopsies from internal carotid plaques were obtained from the Department of Cardiothoracic and Vascular Diseases, "Vita e Salute University", Milan Italy, from patients who underwent surgery between January and December 2002.

Patients aged 64 ± 10 years (83% male) with symptomatic coronary artery diseases (CAD), were subjected to coronary endoartherectomy. Their cardiovascular risk profile included arterial hypertension (blood pressure ≥ 140/90 mmHg,), hypercholesterolemic (≥160 mg/dl low-density lipoprotein cholesterol), 58% were smokers and 33% of them were currently cigarette smoking, 15% were diabetics (≥ 140 mg/dl fasting blood glucose), 4 patients (22%) presented stable angina while the remaining 14 patients (78%) had unstable angina (defined as Braunwald class IIB or greater). Results from pre-operative coronary angiograms were available for all patients from digital storage of the data. The presence of angiographically detectable coronary calcifications or total chronic occlusions was also documented. Decisions for endoartherectomy were on the basis of coronary morphology. Atherosclerotic cylinders of coronary samples were obtained by blunt dissection from the adventitial layer after longitudinal incision of the artery. Immediately after harvesting, samples were stored in sterile tubes for further pathologic examination. All invasive studies were performed after patient written informed consent and were approved by the Ethical Committee of our Institution.

Histological Materials and LCM

Surgically-removed human carotid tissues were rapidly frozen in optimal cutting temperature (OCT) blocks and stored at -80°C. Frozen samples were fixed in10% buffered formalin, were decalcified and finally embedded in paraffin.

The specimens were sectioned with a disposable microtome knife, and the blocks were numbered in sequence starting at the proximal end. Each block was processed through paraffin, sectioned at 8 μm, and then stained in sequence with hematoxylin-eosin. Serial 8 μm-thick cut sections were mounted on a polyethylene foil slide (SL Microtest, Jena, Germany), and stained with HistoGene Staining Solution (Arcturus, Mountain View, CA, USA). Excess water was carefully removed by gently placing a filter on the sample, and all sections were further dried for 15 min at 37°C. Microdissection was performed using a SL-Microcut MMI Cellcut (Molecular Machines & Industries AG, Glattburg, Switzerland).

All plaque components were dissected independently and separately from serial tissue sections by means of an ultraviolet laser that performs dissection of selected tissue areas following precisely a drawn incision path. By this cold ablation, the material to be extracted is never directly exposed to the laser. The microdissected tissue areas were measured by computer-assisted image analyser utilizing the Lucia G software (version 4.82, Nikon, Japan), documented by a 3CCD digital camera, and collected on an adhesive cap of nanotubes for nucleic acid extraction. The microdissected samples were cleanly removed using the MMI Isolation Cap (Molecular Machine & Industries AG, Glattburgh, Switzerland).

DNA Extraction

For each biopsy, the DNA was extracted by using the Nucleospin Tissue kit (Macherey-Nagel GmbH, Düren, Germany), protocol for human tissue, according to the manufacturer's instructions. The final eluate of DNA sample was concentrated by evaporation in 40 μl (final

volume in H_2O). Sterile materials were used throughout, taking strict precautions to avoid contamination. Extraction of DNA and PCR analysis were performed in separate laboratories. All samples were checked for the presence of PCR inhibitors using the LightCycler control kit DNA for human β-globin gene (Roche Diagnostics, GmbH, Germany), according to the manufacturer's instructions.

Real Time PCR and nested PCR

Two microliters of DNA samples were subjected to the quantitative real-time PCR as previously described (4). The *C. pneumoniae* genomic DNA was used at serial dilutions (10^6 to 1 copy number) to generate a standard curve for DNA quantification. The quantitative analysis was made with the LigthCycler quantification software using the cycle threshold of the standard *C. pneumoniae* DNA. Real-time PCR was compared with nested PCR, carried out as previously described (6). In both methodologies 3 negative control reactions were performed with samples containing distilled water / PCR reaction mix. To asses the reproducibility, each clinical sample was analysed twice in 3 independent runs.

Highly-sensitive, nested PCRs were also carried out for the detection of *Helicobacter pylori* and cytomegalovirus (CMV) according to the protocol described by Rassu and colleagues (7).

Statistics

Continuous variables are expressed as mean ± SD (median). Samples were compared using unpaired *t* tests or Mann-Whitney and correlations were assessed using Spearman rank-order correlation coefficients.

RESULTS

Histopathology and LCM

The histological classification of the specimens was performed using criteria for the patient A, B, C lesions established by the American Heart Association (AHA) Committee on Vascular Lesions (8). All carotid endoartherectomy showed macroscopically visible arteriosclerotic lesions with vascular stenosis ranging from 60% to 90%, calcification, presence of macrophages and inflammatory reaction.

Two different types of tissue areas were selected and separately recovered. The area inside the

Table I. *Quantification of C. pneumoniae DNA by Real-time PCR in positive samples.*

N° of Positive plaque 44% (8/18)	Portion localization plaque/ near the plaque	Total CP DNA copy numbers±SD* (plaque/near the plaque)
1	positive/positive	(1900 ±10/50 ±2)
2	positive/positive	(2000 ±15/80 ±3)
3	positive/negative	(700 ±8/0 ±0)
4	positive/negative	(800 ±7/0 ±0)
5	positive/positive	(2200 ±16/48 ±2)
6	positive/negative	(1600 ±10/0 ±0)
7	positive/negative	(900 ±7/0 ±0)
8	positive/negative	(500 ±5/0 ±0)

**the means ± standard deviation of three determinations*

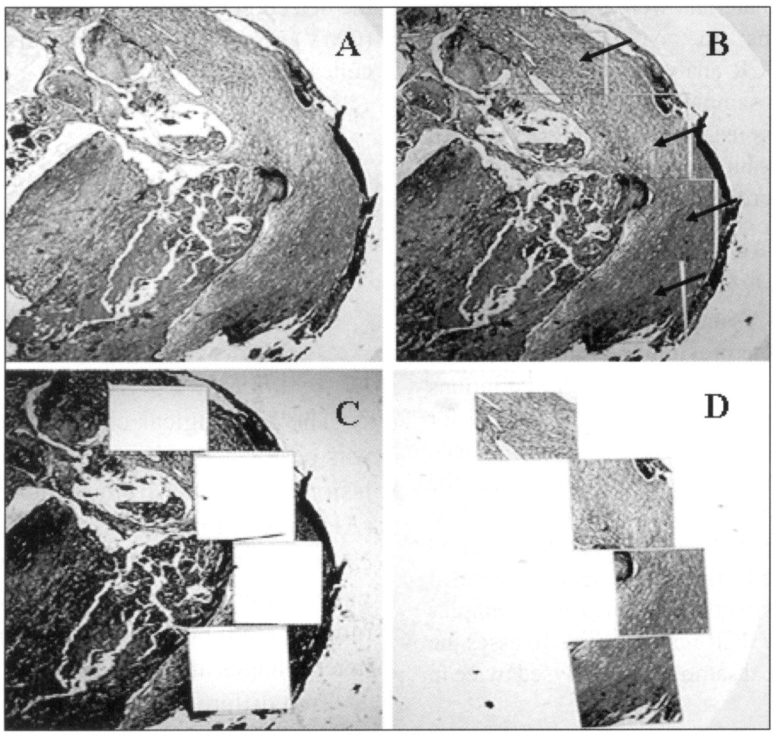

Fig. 1. *Laser capture microdissection procedure.* ***(A)*** *Carotid plaque before laser dissection.* ***(B)*** *Plaque subjected to laser microdissection. Plaque portions are marked with arrows.* ***(C)*** *Tissue remaining after taking away the microdissected areas.* ***(D)*** *Microdissected areas recovered on the nanotube cap*

atherosclerotic plaque consisting of a calcified lipid-rich necrotic core with a predominant cell-free content, lipid and ceroid material. A significantly greater portion of the plaque areas was infiltrated by macrophages with an evident inflammatory reaction. The second tissue portion was the area adjacent to the plaque, consisting in the vessel adventitia. Most of this material was composed of smooth muscle cells, fibroblasts and extracellular connective matrix.

All the above-mentioned microdissected areas were measured and documented by digital camera (Fig. 1 A-D).

Detection and quantitative measurement of C. pneumoniae DNA by Real-time PCR

The real-time PCR assay was assessed for specificity and quantification by construction of a standard curve with purified genomic *C. pneumoniae* DNA. No PCR products or hybridization were detected in the control samples. All samples were positive for the β-globin amplicon, thus no PCR inhibitors were present (data not shown).

The quantitative analysis was performed by the standard curve (inset Fig. 2A) generated from crossing point parameter of the *C. pneumoniae* genomic DNA at dilution series 10^6 to 1 copy (Fig. 2A). As shown in Fig. 2B, the specificity of the assay was confirmed by the melting curve profile of hybridization probes that gave the theoretically expected temperature of 61°C.

A total of 8 plaques from patients with unstable angina (8/14, 57%) were positive in real-time PCR experiments, while none of the plaques from 4 patient with stable angina was found positive to *C. pneumoniae* DNA. The theoretical corresponding DNA copy number was calculated by plotting crossing point of each positive sample versus the standard curve. Results are shown in Table I. In 3 plaques (n° 1, 2 and 5) *C. pneumoniae* DNA was detected in the plaque and the nearby tissue at 1900 ± 10 and 50 ± 2, 2000 ± 15 and 80 ± 3, 2200 ± 16 and 48 ± 2 copy numbers, respectively. The other 5 plaques presented *C. pneumoniae* DNA just within the plaque portion at 700 ± 8, 800 ± 7, 1600 ± 10, 900 ± 7 and 500 ± 5 copy numbers. Remarkably, the bacterial DNA was detected in the portion near

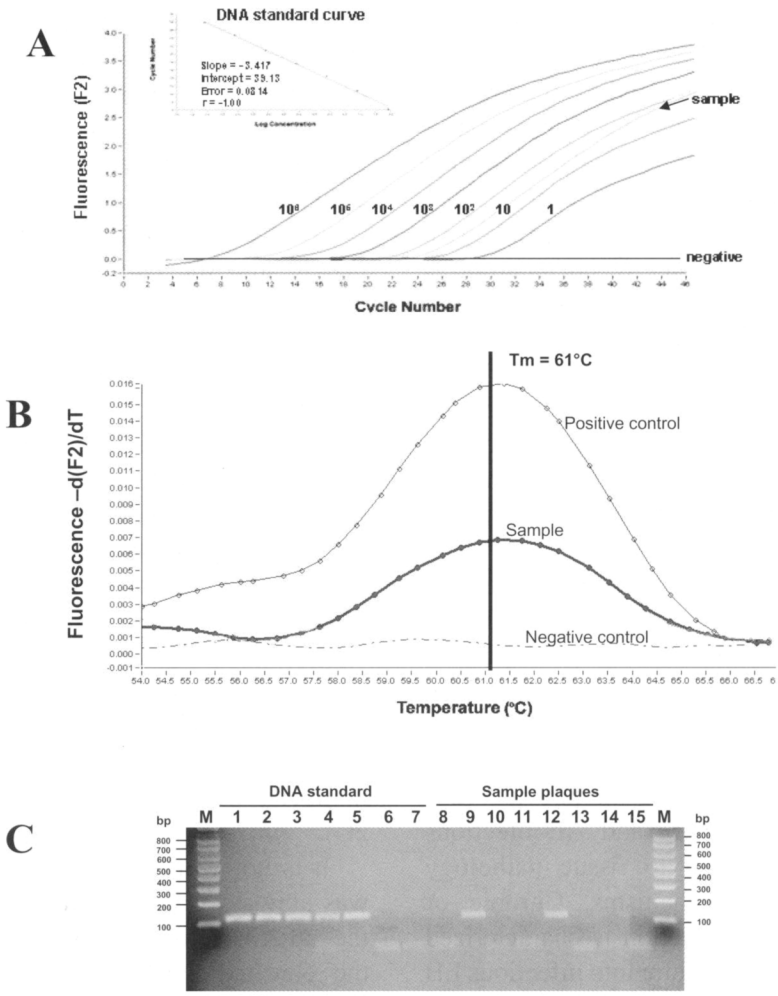

Fig. 2. *C. pneumoniae quantification and melting curve by Real-time PCR.* ***(A)*** *Quantitative analysis and generation of the standard curve.* ***(B)*** *Specific melting curve profile obtained by the FRET hybridization probes. The biopsy sample of plaque n° 4 is also reported as example (arrow).* ***(C)*** *Nested PCR results. Lanes 1-7 standard C. pneumoniae DNA from 10^6 to 1 copy numbers; lanes 8-15 samples from plaque 1 to 8; M is the molecular DNA marker.*

the plaque exclusively in the biopsy samples which presented a higher number of DNA copies (from 1900 to 2200 copy numbers).

DNAs for CMV or *H. pylori* were not found in any plaque, and no apparent association could be found between positives or negatives of Real-time PCR for *C. pneumoniae* and the macro-or microscopic aspect and cell composition of the plaque.

Real-time PCR findings were compared with the nested PCR that amplify the same DNA fragment (4).

PCR products were resolved on 2% agarose gel by electrophoresis and visualized under UV light with ethidium bromide. As shown in Fig. 2C, only two plaque sample (plaque 2 and 5) were positive while all tissue near the plaques were negative (data not shown). As expected, the two positive results were found in samples with the highest DNA copy numbers.

DISCUSSION

Atherosclerosis is considered a lipid-driven inflammatory disease. Several studies indicated a strong heterogeneity of the inflammatory process, consisting of an accumulation of inflammatory cells

in the shoulders and/or upstream of the plaque (9-10).

However, the critical initiator of the inflammatory process has not been identified. Recent studies support the concept that microbes and microbial components might have a part in the inflammatory events leading to plaque formation and /or destabilization (11-12). Among the microbial stimulants, *C. pneumoniae* is now considered to be a potential culprit (13-15), and several studies have detected its presence in the atheromatous plaques (4, 16-19). However, in very few studies, the presence of this bacterium has been quantified and to our knowledge, there was never consideration of the quantitative presence of chlamydial DNA in different areas of the lesion, including the perilesional one.

The contribution of *C. pneumoniae* to the progression of vascular lesions could be a potential risk factor in the multifactorial genesis of the atherosclerosis. The microorganism can infect and modify the physiology of the cell types present in the lung and may transit from the lung to the atheroma via circulating monocytes (20-21). Chlamydial persistence and re-infections are known to be associated to tissue pathology and immunopathologic mechanisms. Chronic or persistent non-productive infection is characterized by abnormal RB that fail to mature into infectious EB (22) and in this circumstance treatment of infected host cells with some antibiotics fails to eradicate the infection but, instead, it promotes chronicity (23). In the context of the persistence, serology does not always correlate with the presence of the organism in atherosclerotic lesions (24). Several authors consider the presence of *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMC) as a valid marker for the presence of the bacteria in the atherosclerotic plaques (25-27). Remarkably, there is discrepancy between *C. pneumoniae* DNA in the PBMCs and in the carotid specimens. In some cases the percentage of individuals who are positive for *C. pneumoniae* in PBMC is significantly lower or higher than the frequency of detection of the microorganism in the plaque. The discordant results may also be attributable to the low sensitivity of the methods, especially in samples with low concentrations of specific DNA, or to the false positive results. In fact, to date no standardized PCR assays are available. Nonetheless, the high sensitivity of our real-time PCR method to detect *C. pneumoniae* has recently been confirmed by Sessa et al. (28) who compared our technique with the *ompA* nested touch-down PCR that was previously investigated by the same group for its reproducibility (29).

A special purpose of our study was to obtain insight into the combined use of LCM and real-time PCR methodologies. We chose particular endoartherectomy tissue samples that are normally problematical for PCR assays since they contain high level calcification, lipids, macrophages and an extensive inflammatory infiltrates.

The LCM allows to examine microscopically and select for specific tissue sections. The real-time PCR offers a very precise quantitative DNA detection even in this type of sample with an expected low DNA amount. As described in a previous work (4), we confirm here, even in the special setting of LCM samples, that real-time PCR is more sensitive than nested PCR. Out of 8 *C. pneumoniae* DNA positive plaques only 2, namely those with the highest copy numbers, were also positive in nested PCR (Fig. 2C).

It is noteworthy that here *C. pneumoniae* DNA was almost exclusively found in the portion inside the atherosclerotic lesion. Only the plaques with the elevated DNA copy numbers presented also the bacterial DNA with low copy numbers outside the lesion, in the vessel adventitia. This result may be attributed to the microbial spreading from the lesion during LCM rather than to an effective local infection. Although no correlation was found between chlamydial DNA copy number and histopathological severity of lesions, this remains an open issue for further investigation since the plaques examined and the fraction of them which were positive for chlamydial DNA were too few to attempt any kind of statistical examination.

The large amount of chlamydial DNA that we found in some sections might support the hypothesis of a resident and consistent *C. pneumoniae* infection. It is not possible, however, to speculate on the nature of the infection, be it productive, chronic or latent status. Serological tests are controversial and for some authors there is poor correlation between *C. pneumoniae* DNA detection in atherosclerotic lesions and serology (24, 26).

To clarify the role of *C. pneumoniae* in atheromatous lesion generation and/or destabilization, genomic and proteomic tools are needed to be assessed. Of these methods, the combination of highly sensitive PCR analysis based on Real-time PCR with LCM provides considerable advantages. Actually, within *in situ* biopsy tissue, it may be possible to study gene expression in each of the characteristic tissue phases and forms of *C. pneumoniae* infection.

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