

## Detection of *Chlamydia pneumoniae* (*Chlamydophila pneumoniae*) DNA in atherosclerotic plaques and its molecular analysis in northern Greece

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**ABSTRACT:** *Objectives:* *C. pneumoniae* responsible for respiratory tract infections has also been associated with chronic diseases such as atherosclerosis.

The aim of the present study is the detection of *C. pneumoniae* DNA in various atherosclerotic arteries by a sensitive and specific PCR. In order to investigate whether there is a relation between a specific type and atherosclerosis, genotyping was performed.

*Methods:* The study group consisted of 122 atherosclerotic plaques from patients (mean age 68.4, range 50-89 years old, 95 males and 25 females) with severe atherosclerosis. *C. pneumoniae* DNA was detected in atherosclerotic plaques by nested «Touchdown» PCR. A second PCR targeting the *ygeD-urk* intergenic region was performed and PCR products were sequenced.

*Results:* 12.3% of the specimens were positive for *C. pneumoniae*. Detection rates in specimens of carotid, abdominal, and femoral arteries were 12%, 15.6%, and 10%, respectively. ( $p = \text{NS}$ ). 14 strains were found to have 100% homology with J138, AR39 and TW-183, while one strain had a 23 bp invertible region and revealed 100% homology with the CWL029.

*Conclusion:* Overall, 15/122 (12.3%) atherosclerotic specimens from patients were positive for *C. pneumoniae*. The strains detected belong to two different types designated as genotype I and II. Genotype I was the prevalent and only one strain had the reverse orientation of the 23bp region in northern Greece.

*Key Words:* *Chlamydia pneumoniae*, *Atherosclerosis*, *PCR*, *Molecular analysis*.

### INTRODUCTION

*Chlamydia pneumoniae* (*Chlamydophila pneumoniae*) is a Gram negative intracellular bacterium responsible for acute upper and lower respiratory tract infections<sup>1</sup> and for many years scientific interest has been focused on its possible association with chronic diseases such as atherosclerosis<sup>2</sup>. The first association between *C. pneumoniae* and coronary artery disease (CAD) was based on serologic studies in Finland in which high titers of IgG and IgA antibodies to *C. pneumoniae* were found significantly more often in men with myocardial infarction and chronic CAD than in age-matched randomly selected controls<sup>3</sup>. Since then a number

of observational studies followed demonstrating the presence of *C. pneumoniae* by multiple methods such as electron microscopy, immunocytochemistry and polymerase chain reaction (PCR) not only in coronary but in various atherosclerotic arteries. However, there is as yet no conclusive evidence about the role of *C. pneumoniae* in atherosclerosis.

The aim of this study was the detection of *Chlamydia pneumoniae* DNA in atherosclerotic plaques by nested «Touchdown» PCR targeting the *OmpA* gene. Nested PCR was used because it is considered more sensitive than single-step PCR, and «Touchdown» PCR increases specificity by allowing the initial prim-

**Table 1.** Detection of *C. pneumoniae* DNA in atherosclerotic plaques.

Sample origin	No of samples tested	PCR positive	
		No.	%
Carotid artery	50	6	12
Abdominal aorta	32	5	15.6
femoral	40	4	10
Total	<b>122</b>	<b>15</b>	<b>12.3</b>

er-template hybridization events to occur at annealing temperatures that are greater than the optimum annealing temperature. In order to investigate whether there is a relation between a specific type of *C. pneumoniae* and atherosclerosis, genotyping was performed.

### PATIENTS

The study group consisted of 122 patients (mean age 68.4, range 50-89 years old, 95 males and 25 females) with severe atherosclerosis and therefore underwent elective surgery for atherosclerotic plaque removal from May 2004 to April 2006. An equal number of atherosclerotic plaques (carotid artery, n = 50; femoral artery, n = 40; abdominal aorta, n = 32) were immediately transferred to the laboratory (15-20 min), where they were homogenized with the use of mortar and liquid nitrogen. The homogenized samples were given a code number, divided into four aliquots and stored at -70°C for PCR studies.

### METHODS

#### *DNA extraction*

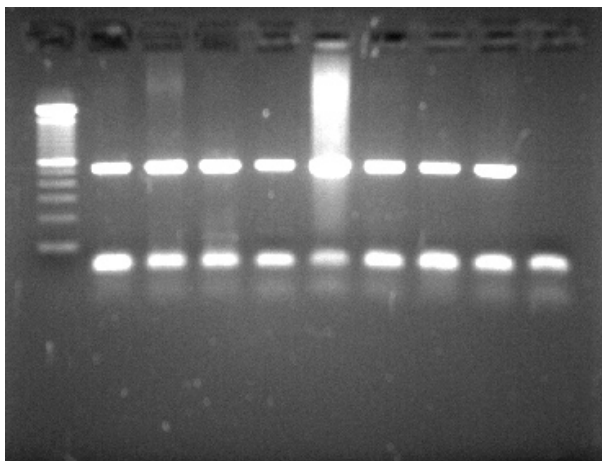
DNA extraction from the atherosclerotic plaque was performed using the DNA mini kit of Qiagen (Greece, BioAnalytica S.A.) according to the manufacturer's recommendations. Briefly the method uses a QIAamp spin column to which DNA binds in the presence of buffer AL and ethanol. Two wash steps, in which AW1 buffer and AW2 buffer succeed each other, are performed to remove contaminants. AE buffer is finally used for elution of the DNA from the spin column.

#### *Detection of C. pneumoniae ompA gene*

The presence of *C. pneumoniae* in the 122 atherosclerotic plaques was detected by a nested «Touchdown» PCR method. The major outer protein genes (*ompA*)

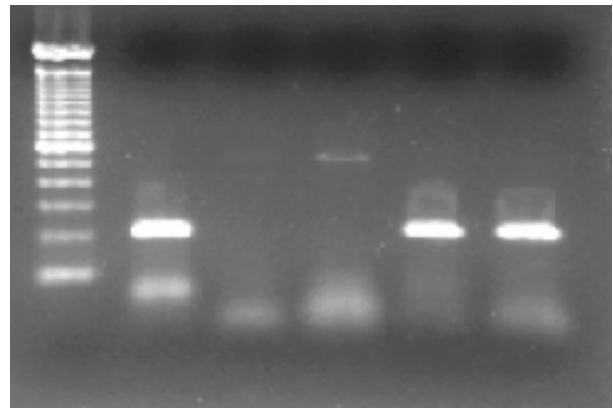
of *C. pneumoniae* were chosen as the target for amplification as previously described by Tong and Sillis [4]. The external primers, CP1: 5' TTA CAA GCC TTG CCT GTA GG3' and CP2: 5' GCG ATC CCA AAT GTT TAA GCG 3' amplified a 333 base pair product, and the internal primers CP6: 5' TTA TTA ATT GAT GGT ACA ATA 3' and CPD: 5' ATC TAC GGC AGT AGT ATA GTT 3' amplified a 207 base pair product. The first stage of PCR was performed in a 100µl final volume containing: 10 µl of the extracted DNA, PCR buffer 10X (200mM Tris-HCl, pH 8.4 and 50mM KCl), 0.4 µM each of outer primers, 1.5 mM MgCl<sub>2</sub>, 200 µM each deoxyribonucleotide (dATP, dTTP, dCTP and dGTP) and 2.5U Taq polymerase (Invitrogen, UK). Touchdown PCR was performed in which the annealing temperature was lowered 1° C every two cycles from 64° C until touching down to 55° C, at which temperature 30 more cycles were programmed. The denaturation and extension temperature were constant at 94° and 72° C, respectively. The holding time of each temperature was 1 minute. In order to check the quality of the extraction process and the possible presence of PCR inhibitors, b-Globine specific primers were used in each reaction, KM29: 5' GGT TGG CCA ATC TAC TCC CAG G 3' and RS42: 5' GCT CAC TCA GTG TGG CAAAG 3', which amplified a 536 base pair product (Figure 1).

The PCR products amplified by the external primers were diluted 10 times in DNase free water and 10µl of it was transferred to a PCR reaction mix for the second stage of amplification using the internal primers. The PCR reaction mix was the same as the first one, with two exceptions; 1 µM of each of the internal primers was used and the MgCl<sub>2</sub> concentration was increased to 3 mM. This PCR second stage PCR consisted of 30 cycles of 94°, 50° and 72°C each for 1 minute.



L 64 65 66 67 68 69 70 71 (-)

**Figure 1.** Representative agarose gel electrophoresis of first stage PCR. L: Molecular size ladder (100bp), 64, 65, 66, 67, 68, 69, 70, 71: samples of atherosclerotic plaques, (-): negative control. All show a 536 bp fragment for  $\beta$ -Globin.



L (+) (-) 27 16 37

**Figure 2.** Representative agarose gel electrophoresis of second stage PCR. L: Molecular size ladder (100bp),(+): positive control, (-): negative control, 27, 16, and 37: atherosclerotic plaque samples.

Both first and second stage products were analyzed in 2% gel electrophoresis. Every sample revealed a  $\beta$  - Globine 536 base pair in the first stage PCR and after second PCR the positive results revealed a 207 base pair fragment (Figure 2). Positive and negative controls were included in every PCR run.

#### Detection of *ygeD-Urk gene*

The positive samples for *C. pneumoniae* presence were further studied. It has been reported that *C. pneumoniae* strains differ in the plasticity zone between the *ygeD* and *urk* genes by the orientation of a 23 bp sequence. Therefore in this study a 568 bp fragment targeting this region was amplified using a nested PCR. The external primers were *ygeD*1: 5'GTT AGG GTG GTT TTC CCA GC 3' and *ygeD*2: 5'GAG ATA ACG ATT CTG AGG CC 3' and the internal, *ygeD*3: 5' CCA GAA CCT CCT GTA ATT CC3' *ygeD*4: 5'GCT GTT CTA CAA GCA AAT CAC CC 3'. The first stage PCR was performed in a final volume of 50  $\mu$ l containing: 5 $\mu$ l DNA, PCR buffer 10X (200 mM Tris-HCl, pH 8.4 and 50 mM KCl), 0.5  $\mu$ M from each primer, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M of each deoxyribonucleotide (dATP, dTTP, dCTP and dGTP) and 2.5 U *Taq* polymerase (*Taq* DNA polymerase, recombinant, Invitrogen, UK). PCR conditions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C, annealing at 56°C, and

extension at 72°C, all for 1 min, and a final extension at 72°C for 10 min.

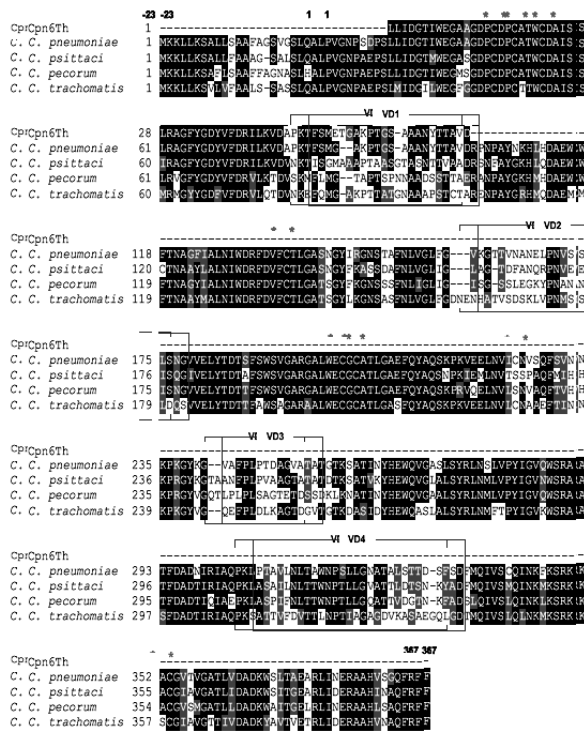
The second stage PCR was performed in a final volume of 50  $\mu$ l containing 1  $\mu$ l of the product from the first PCR, PCR buffer 10X (200 mM Tris-HCl, pH 8.4 and 50 mM KCl), 0.5  $\mu$ M from each primer, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each deoxyribonucleotide (dATP, dTTP, dCTP, and dGTP) and 2,5 U *Taq* polymerase (*Taq* DNA Polymerase, recombinant, Invitrogen, UK). The PCR conditions remained the same except from the annealing temperature that was modified to 51°C. The samples revealed in electrophoresis a 371 bp fragment.

All products were analyzed in 2% agarose gel electrophoresis with ethidium bromide staining.

#### Sequencing of PCR products and analysis

Both PCR products were purified using the commercial kit Chargeswitch PCR cleanup kit (Invitrogen, UK) according to the manufacturer's instruction. Purified PCR products were sequenced (Lark Technologies, Congenics Ltd, Essex, UK) using the CP6: 5' TTA TTA ATT GAT GGT ACA ATA 3' and CPD: 5' ATC TAC GGC AGT AGT ATA GTT 3' primers, and *ygeD*3: 5' CCA GAA CCT CCT GTA ATT CC3' and *ygeD*4: 5'GCT GTT CTA CAA GCA AAT CAC CC 3', for each amplified product.

Sequences were further analyzed using bioin-



**Figure 3. Alignment of CpnThessaloniki *ompA* with other *ompA*.** The deduced amino-acid sequence for CpnThessaloniki *ompA* was compared to *C. pneumoniae* J138 (NP\_300751), *C. psittaci* FPN (Q00087), *C. pecorum* LW613 (CAD29328) and *C. trachomatis* L2 (P06597). The black and grey shading indicates identical and similar amino-acids in properties, respectively. The four VD's are boxed, while the conserved cysteines are indicated with an asterisk.

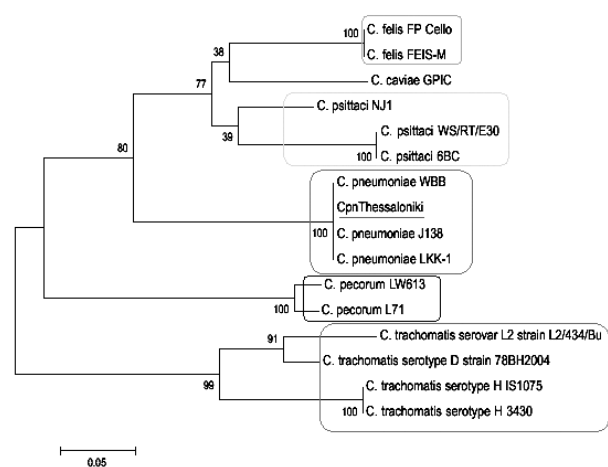
formatic tools. In particular, related sequences were found using the BLAST tool through the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>), while the multiple sequence alignments were generated with ClustalW from the European Bioinformatics Institute. Furthermore, the phylogenetic and molecular evolutionary analysis was conducted using MEGA3.1.

*Statistical analysis*

Statistical analysis for discrete variables was made by the  $\chi^2$  test. After controlling for normal distribution, continuous variables were analyzed by the Mann-Whitney test.  $P < 0.05$  was considered significant.

**RESULTS**

Overall, 15 of 122 (12.3%) atherosclerotic speci-

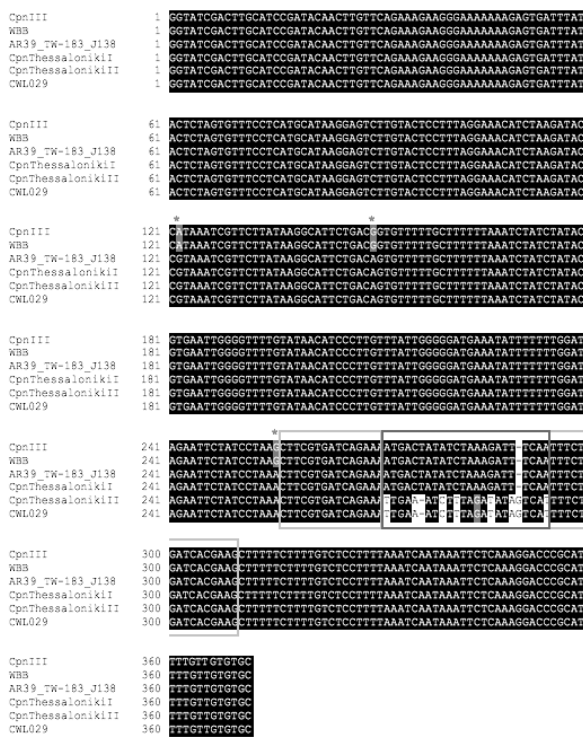


**Figure 4. Phylogenetic relatedness of CpnThessaloniki *ompA* to other known *ompA*.** Deduced amino acid sequences were analyzed by Neighbor-Joining (NJ) using MEGA 3.1. The cladogram was generated from an alignment of CpnThessaloniki with *C. felis* FP Cello (AAK00239), *C. felis* FEIS-M (AAO24108), *C.aviae* GPIC (AAK00263), *C. psittaci* NJ1 (AAK00247), *C. psittaci* WS/RT/E30 (AAX09977), *C. psittaci* 6BC (CAA40300), *C. pneumoniae* WBB (ABC86832), *C. pneumoniae* J138 (NP\_300751), *C. pneumoniae* LKK-1 (AAT68197), *C. pecorum* LW613 (CAD29328), *C. pecorum* L71 (AAK00261), *C. trachomatis* serovar L2 strain L2/434/Bu (P06597), *C. trachomatis* serotype D 78BH2004 (ABE02281), *C. trachomatis* serotype H IS1075 (AAL61634) and *C. trachomatis* serotype H 3430 (CAJ46707).

mens from patients were positive for *C. pneumoniae* by PCR (table 1). Detection rates of *C. pneumoniae* in arterectomy specimens of carotid, abdominal, and femoral arteries were 12%, 15.6%, 10 % respectively. The detection rates comparing the various atherosclerotic tissues were not significantly different ( $p = 0.64$ ,  $p = 0.76$ ,  $p = 0.47$ ).

The *ompA* region amplified (207 bp) was sequenced of the 15 positive samples and showed that they were identical and revealed 100% homology to the *C. pneumoniae* J138 strain (NP\_300751) and less with other species of chlamydophila genus *C. psittaci* FPN (Q00087), *C. pecorum* LW613 (CAD29328) and *C. trachomatis* L2 (P06597). The alignment of the amino acid sequence of the detected *ompA* with other known *ompA* sequences of the genus is presented in Figure 3. The amino acid sequence is relatively conserved

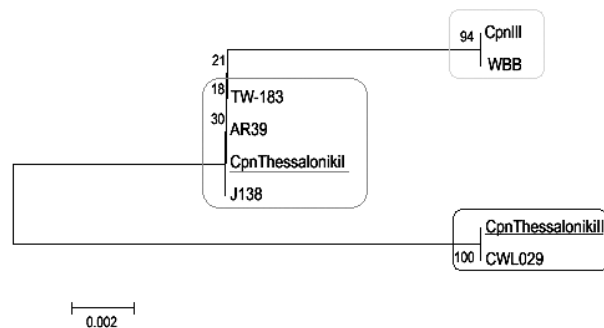




**Figure 5.** Alignment of the fragment ygeD-urk gene of CpnThessaloniki I and II with various *C. pneumoniae* isolates. The deduced amino-acid sequence for CpnThessaloniki I and II ygeD-urk region was compared to J138 (BA000008), TW-183 (AE017159), AR39 (AE002161), CWL029 (AE001363) WBB (DQ463439) and CpnIII (AY427827). The black and grey shading indicates identical and similar amino-acids in properties, respectively. The amino-acid residues in the blue box are a 23 bp inverted sequence, while those in orange boxes are the 15 bp inverted repeats. The amino-acids indicated with an asterisk are diversified in WBB and CpnIII.

between the species of chlamydia, but there can be identified four distinct regions (variable domains, VDs) with lower conservations. The *ompA* domain isolated contains a highly conserved region, as well as the VD1 domain (Figure 3). Furthermore, three out of seven conserved cysteins responsible for the sulphid bond were identified in our sequence (Figure 3). The phylogenetic relatedness of the isolated amino acid sequence with other known chlamydia *ompAs* is presented in Figure 4. The strain is grouped with other strains of *C. pneumoniae* like J138, WBB and LKK-1. Four further groups were presented (Figure 4).

As far as it concerns the intergenic spacer region between ygeD and urk genes (371 bp) used for the



**Figure 6.** Phylogenetic relatedness of the fragment ygeD-urk gene of CpnThessaloniki I and II with various *C. pneumoniae* isolates. Deduced amino acid sequences were analyzed by Neighbor-Joining (NJ) using *MEGA* 3.1. The cladogram was generated from an alignment of CpnThessaloniki I and II with J138 (BA000008), TW-183 (AE017159), AR39 (AE002161), CWL029 (AE001363) WBB (DQ463439) and CpnIII (AY427827).

genotyping, 14 of our strains were identical and 100% homologous to J138, AR39 and TW-183 strains, while one sample (Carotid atherosclerotic plaque, 37) had the 23 bp invertible region and revealed 100% homology with the CWL029 strain. The alignment of the amino acid sequences is shown in Figure 5. This finding indicates that the strains isolated from the samples belong into two different types. The phylogenetic relationship of the amino acid sequences isolated from different samples with known ygeD-urk regions supports this finding (Figure 6).

## DISCUSSION

The «infectious» hypothesis as a causal role of athero-

sclerosis is surprisingly old since infectious agents have been proposed to participate in the disease since the late 1800s and early 1900s<sup>5</sup>. In 1908 Sir William Osler suggested that “acute infections” including scarlet fever, measles, diphtheria, smallpox and influenza might be responsible for atherosclerosis<sup>6</sup>. In a 1911 paper, Frothingham<sup>7</sup> reported that «acute infectious diseases may cause some pretty general lesion throughout the arterial system by the infecting organisms. The exact nature of these lesions in human cases and their final result, have not been so well worked out». A century later, scientists have not yet come to an agreement about this association. Meanwhile, Fabricant et al<sup>8</sup> were the first researchers to experimentally induce atherosclerosis in chickens by inoculation with a chicken herpesvirus that induces fibrolymphomatosis. The induction of atherosclerotic changes was prevented by vaccination. Since then, a number of microorganisms have been implicated in atherosclerosis including *Cytomegalovirus* (CMV), *Herpes simple virus* (HSV), *H. pylori* and *C. pneumoniae*, all possessing an important similarity as they are all obligate intracellular pathogens capable of maintaining a chronic persistent state in the host<sup>5,9-11</sup>.

The present study works on the detection of *C. pneumoniae* DNA in atherosclerotic plaques. Since Saikku et al.<sup>3</sup> demonstrated serological evidence of an association of *C. pneumoniae* with chronic coronary heart disease and acute myocardial infection, the research on the infection hypothesis and particularly by the specific microorganism has significantly boosted. Many research groups using various techniques have tried to link *C. pneumoniae* to chronic diseases such as atherosclerosis.

However, recently, serious doubts have been raised about the role of *C. pneumoniae* in atherosclerosis not only from the experimental studies<sup>12,13</sup> but primarily due to the negative results of large antibiotic trials investigating the efficacy of antibiotic treatment for the secondary prevention of cardiovascular events<sup>14</sup>. These results have suggested that the infection hypothesis is possibly wrong and that the microorganism is just an innocent bystander in the vascular wall.

In our study we demonstrated the presence of *C. pneumoniae* in 12.3%, (15/122) atherosclerotic samples analyzed by PCR. This low detection rate of *C.*

*pneumoniae* DNA in the atherosclerotic samples is probably due to the presence of low density of the microorganism in the atherosclerotic lesion or to the high specificity PCR. In other studies using different PCR methods, the detection rate of *C. pneumoniae* DNA varies between 0-75%. For example in 1993, Kuo et al detected *C. pneumoniae* DNA by PCR in 43.3%, 13/30, of coronary atheromatic arteries in autopsy cases from South Africa<sup>15</sup>. In 1996, Blasi et al detected the bacterial DNA in 51%, 26/51 of the atheromatic plaques tested<sup>16</sup>, while Mass et al. also detected it in atherosclerotic coronary arteries in a ratio of 30%, 21/70<sup>17</sup>. In 1999, Jantos et al detected the DNA of *C. pneumoniae* in 8% of patients with coronary artery disease<sup>18</sup>, while Nadrchal et al. found it in 28 % of the atheromatic carotid plaques<sup>19</sup>. Moreover, Farsak et al, in 2000, detected the microorganism in a ratio of 26 %<sup>20</sup>, while Cochrane et al., in 2002 in Australia, detected it in 2 out of 28 atheromatic plaques (7.14 %)<sup>21</sup>. Undoubtedly, in 2003, Zamorano et al. using the same PCR method found *C. pneumoniae* DNA in 75% of patients with unstable angina undergoing bypass surgery<sup>22</sup>.

Although, few studies haven't succeeded to detect *C. pneumoniae* DNA in any atherosclerotic plaque and reject the role of the microorganism in atherosclerosis, the presence of *C. pneumoniae* DNA in our patients, as in previous studies mentioned before, preserves the interest of the participation of *C. pneumoniae* in atherosclerosis<sup>13,23</sup>.

According to the sequence analysis of the *ompA* region amplified, our strains were identical and revealed 100% homology with the respiratory strain J138.

A recent study comparing the strains CWL029 and AR39 reported a differentiation in the plasticity zone between the *ygeD* and *urk* genes by the orientation of a 23 bp sequence<sup>24</sup>. This region has been used in the sequencing of *C. pneumoniae* by other researchers as well, although, this kind of studies are quite recent and rare<sup>25,26</sup>. The 23 bp invertible region in the *C. pneumoniae* plasticity zone is in the forward orientation in 3 respiratory reference strains and one ocular (J138, AR39, IOL207, TW183); and in the reverse orientation in one of the strains, (CWL029). Further molecular analysis of our strains based on the orientation difference of the 23 bp region between the *ygeD*-

urk genes revealed that 14/15 strains were identical to each other and to 3 respiratory and one ocular strain studied, designated CpnThessaloniki I. One strain, designated CpnThessaloniki II had the reverse orientation of the 23 bp region and was 100% identical to the respiratory reference strain CWL029.

Concluding, *C. pneumoniae* DNA was detected in 15 out of 122 (12.3%) atherosclerotic specimens from patients undergoing elective surgery. This result pre-

serves the hypothesis that the microorganism could be a possible etiological co-factor in the pathogenesis of atherosclerosis.

The strains detected in the samples belong to two different types designated as CpnThessaloniki I and II. CpnThessaloniki I was the prevalent and only one strain had the reverse orientation of the 23bp region in northern Greece. All our strains were 100% homologous to other respiratory and ocular strains.

## Ανίχνευση DNA του Χλαμυδίου της πνευμονίας (Χλαμυδόφιλα της πνευμονίας) σε αθηρωματικές πλάκες και η μοριακή του ανάλυση στη βόρεια Ελλάδα

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**ΠΕΡΙΛΗΨΗ:** Το Χλαμύδιο της πνευμονίας είναι αίτιο λοιμώξεων του αναπνευστικού αλλά έχει συσχετισθεί και με την παθογένεια χρόνιων νοσημάτων όπως η αθηροσκλήρωση.

Σκοπός της παρούσας μελέτης είναι η ανίχνευση του DNA του Χλ. της πνευμονίας σε αθηρωματικές πλάκες από διάφορα αγγεία με τη χρήση PCR υψηλής ειδικότητας και ευαισθησίας. Για την περαιτέρω διερεύνηση της σχέσης κάποιου τύπου του μικροοργανισμού με την αθηρωμάτωση, έγινε γενετική τυποποίηση.

**Μέθοδοι.** Εξετάστηκαν 122 αθηρωματικές πλάκες από ισάριθμους ασθενείς. (μέσος όρος ηλικίας 68,4, ηλικιακό εύρος 50-89 έτη, 95 άρρενες και 25 θήλειες) με προχωρημένου βαθμού αθηροσκλήρωση. Για την ανίχνευση του DNA του μικροοργανισμού εφαρμόστηκε η nested Touchdown PCR και για την περαιτέρω ανάλυση, μια nested PCR με εκκινητές που στοχεύουν στην ygeD-urk περιοχή. Έγινε αλληλούχιση και ανάλυση των προϊόντων των PCR.

**Αποτελέσματα:** Στο 12,3% των δειγμάτων ανιχνεύθηκε DNA του μικροοργανισμού. Η αναλογία ανίχνευσης ανάλογα με το αγγείο προέλευσης της αθηρωματικής πλάκας, καρωτίδες, κοιλιακή αορτή και μηριαίες αρτηρίες ήταν 12%, 15,6% και 10% αντίστοιχα. ( $p = NS$ ). 14 από τα στελέχη μας είχαν 100% ομολογία με τα στελέχη J138, AR39 και TW183 και ένα στέλεχος είχε στην ygeD-urk περιοχή, ένα τμήμα 23 ζβ με ανάστροφο προσανατολισμό και εμφάνισε 100% ομολογία με το στέλεχος CWL029.

**Συμπεράσματα:** Στα 15/122 (12,3%) δείγματα ανιχνεύθηκε γενετικό υλικό του μικροοργανισμού. Τα ανιχνευθέντα στελέχη κατηγοριοποιήθηκαν σε δύο γονότυπους, I και II. Ο γονότυπος I ήταν ο επικρατέστερος και ένα μόνο στέλεχος είχε το τμήμα των 23ζβ ανάστροφο.

**Λέξεις Κλειδιά:** Χλαμύδιο της πνευμονίας, Αθηροσκλήρωση, PCR, Μοριακή ανάλυση.

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