**Chlamydia pneumoniae** is Prevalent in Symptomatic Coronary Atherosclerotic Plaque Samples Obtained From Directional Coronary Atherectomy, but its Quantity is Not Associated With Plaque Instability: An Immunohistochemical and Molecular Study

Tomoyuki Otani¹,², Kensaku Nishihira³, Yoshinao Azuma⁴, Atsushi Yamashita⁵, Yoshisato Shibata³, Yujiro Asada³,⁶ and Kinta Hatakeyama¹,⁵

¹Department of Pathology, National Cerebral and Cardiovascular Center, Suita, Osaka, Japan. ²Department of Pathology, Kindai University Faculty of Medicine, Osaka-Sayama, Osaka, Japan. ³Department of Cardiology, Miyazaki Medical Association Hospital, Miyazaki, Japan. ⁴Molecular Biochemistry Lab, Biology-Oriented Science and Technology, Kindai University, Kinokawa, Wakayama, Japan. ⁵Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan. ⁶Department of Pathology, Miyazaki Medical Association Hospital, Miyazaki, Japan.

**ABSTRACT**

AIM: To clarify whether there is any association between the extent of *Chlamydia pneumoniae* (*C. pneumoniae*) infection and plaque instability or post–directional coronary atherectomy (DCA) restenosis, we determined the frequency of *C. pneumoniae* infection and its localization in symptomatic coronary atherosclerotic plaques using specimens obtained from DCA.

METHODS AND RESULTS: Immunohistochemistry (IHC) and real-time polymerase chain reaction (RT-PCR) revealed the existence of *C. pneumoniae* in all 50 specimens of coronary atherosclerotic plaques obtained by DCA. *C. pneumoniae*–positive cell ratio determined with IHC or copy numbers of *C. pneumoniae* DNA detected by RT-PCR did not differ significantly between patients with stable angina pectoris and those with acute coronary syndrome (IHC: 16.4 ± 7.6% vs 18.0 ± 7.1%, P = .42; RT-PCR: no. of cases with high copy numbers 12/25 vs 10/25, P = .78), or between patients with subsequent post-DCA restenosis and those without (IHC: 17.1 ± 8.0% vs 18.0 ± 7.4%, P = .74; RT-PCR: 5/12 vs 10/21, P = 1.00).

CONCLUSIONS: *C. pneumoniae* was highly prevalent in coronary atherosclerotic plaques of patients who underwent DCA. However, the extent of *C. pneumoniae* infection in coronary atherosclerotic plaques was not associated with plaque instability or post-DCA restenosis.

KEYWORDS: Coronary artery atherosclerosis, *Chlamydia pneumoniae*, immunohistochemistry, PCR, directional coronary atherectomy

**Introduction**

When inflammation plays a major role in a disease, it is often a question of whether or to what extent this inflammation is caused by an exogenous pathogen. Many diseases (eg, sarcoidosis,⁷ multiple sclerosis,⁸ inflammatory bowel disease, etc) have a long history of this type of controversy. Atherosclerosis is no exception. In the early 20th century, scarlet fever and measles were cited among others as putative causes of atherosclerotic lesions. More recently, cytomegalovirus, herpes simplex virus, and *Helicobacter pylori* attracted attention.⁴ But it is *Chlamydia pneumoniae* (*C. pneumoniae*) that has been regarded as the most promising candidate in the last few decades. In 1988, Saikku et al reported that raised serum anti-*C. pneumoniae* IgG and IgA titers were associated with coronary heart disease.⁵ Subsequently, *C. pneumoniae* organisms were detected in coronary atherosclerotic lesions using immunohistochemistry (IHC), polymerase chain reaction (PCR), and electron microscopy.⁶ In an animal study, *C. pneumoniae* has been shown to promote atherosclerosis in rabbits and this infection-induced acceleration could be deterred with antibiotic treatment.⁷ Although the enthusiasm for infectious etiology has somewhat abated after large clinical trials involving more than 15,000 patients in total could not prove the efficacy of antibiotic therapy targeting *C. pneumoniae* for the prevention of severe sequelae of coronary artery disease⁸–¹⁰, *C. pneumoniae* remains an interesting and underexplored topic in the pathogenesis of atherosclerosis.
In this study, we examined the frequency, extent, and tissue localization of *C. pneumoniae* infection in coronary atherosclerotic plaques using specimens obtained from directional coronary atherectomy (DCA). We also investigated the possible association between the extent of *C. pneumoniae* infection and plaque instability or post-DCA restenosis.

**Materials and Methods**

**Case selection**

Fifty patients who underwent DCA at Miyazaki Medical Association Hospital (Miyazaki, Japan) were included in the study. Twenty-five patients were diagnosed with stable angina pectoris (SAP), 21 with unstable angina pectoris (UAP), and 4 with acute myocardial infarction (AMI). Patients with angina at rest, new-onset angina of less than 1 month’s duration, or progressive angina were diagnosed with UAP. UAP and AMI were combined as an acute coronary syndrome (ACS) for subsequent analysis. DCA samples were obtained from left anterior descending coronary artery (n = 41), left circumflex coronary artery (n = 2), left main trunk (n = 1), and right coronary artery (n = 6). Thirty-three of 50 patients underwent follow-up coronary angiography and the presence or absence of restenosis was recorded. Clinical information was retrieved from medical records. Risk factors for coronary artery disease included hypertension, hyperlipidemia, hyperuricemia, diabetes, smoking, obesity (body mass index >30 kg/m²), and family history of coronary artery disease. All patients provided written informed consent to participate in the study. This study was approved by the Human Investigation Review Committee of Miyazaki Medical Association Hospital (Approval No. 15) and conformed with the principles outlined in the Declaration of Helsinki.

**Immunohistochemistry**

Tissue samples obtained by DCA were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4-µm thick) were stained with hematoxylin and eosin for morphological studies and serial sections were examined by IHC. IHC was performed as previously described. Briefly, sections were incubated with 3% hydrogen peroxide in methanol for 20 minutes and then with primary antibodies. Intervening washes with PBS were followed by incubation with EnVision+ (Dako, Japan) for 30 minutes at room temperature. After further washes, the sections were incubated with 0.05% 3,3′-diaminobenzidine containing hydrogen peroxide and counterstained with Mayer’s hematoxylin. Two primary antibodies, RR402 and CF2, were used to identify *C. pneumoniae* on tissue sections. RR402 is reported to be specific to *C. pneumoniae*, while CF2 is Chlamydia genus-specific. Immunostaining with primary antibodies against muscle actin (clone HHF35), CD68 (clone PGM-1), CD34, and CD3 were used to aid in identifying smooth muscle cells, macrophages, endothelium, and T-lymphocytes, respectively. To determine the ratio of *C. pneumoniae*-infected cells, 3 observers (TO, AY, KH) manually counted the number of positively and negatively stained nuclei of any type of nucleated cells in 5 high-power fields per specimen, as previously described. The results are reported as the ratio of positively stained nucleated cells.

**Real-time PCR analysis**

DNA was extracted from formalin-fixed paraffin-embedded tissue specimen using the TaKaRa DEXPAT kit (Takara, Shiga, Japan) according to the manufacturer’s instructions. PCR was performed with Qiagen Quantitect SYBR Green PCR (Qiagen, Hilden, Germany) with primers targeting *C. pneumoniae* repetitive sequences and human ribosomal DNA (rDNA). PCR products were subjected to 2% agarose gel electrophoresis. Primer sequences were 5′−CACAGATTCATA ATGCAAGTA−3′ and 5′−TCAGTAAGAGCACAACCAAG AACTAAA−3′ for *C. pneumoniae* repetitive sequence and 5′−CTAATACATGCGACGGCCGC−3′ and 5′−GGGGT GTGCGATCGCCCCAG−3′ for human rDNA. The concentrations of *C. pneumoniae* repetitive sequence DNA were normalized to those of human rDNA and were categorized as high or low.

**Statistical analysis**

Categorical variables were compared using the Fisher’s exact test and numerical variables with the Welch t-test. A *P* value of <.05 was considered significant. Statistical analyses were performed using R software (version 3.5.2, https://www.R-project.org/).

**Results**

**Patient characteristics**

The clinical characteristics of patients are summarized in Table 1. Smoking was more frequent in the ACS group (6/25) than in the SAP group (17/25) (*P* < .01, Fisher’s exact test); other risk factors for coronary artery disease did not differ significantly between the SAP and ACS groups. Follow-up coronary angiography revealed restenosis in 12 of 33 patients who underwent this procedure.

**Immunohistochemical detection and localization of *C. pneumoniae***

Figure 1 shows representative microphotographs of immunohistochemical staining for *C. pneumoniae* in coronary atherosclerotic plaque specimens obtained by DCA. Many macrophages and some smooth muscle cells stained positive. No positive staining was observed in lymphocytes or endothelial cells. *C. pneumoniae*-positive cell ratio did not differ significantly between the SAP and ACS groups (16.4 ± 7.6% vs 18.0 ± 7.1%, *P* = .42), or between patient groups with and without subsequent post-DCA restenosis (17.1 ± 8.0% vs 18.0 ± 7.4%, *P* = .74) (Figure 2).
PCR detection and quantification of *C. pneumoniae*

*C. pneumoniae* repetitive sequence DNA was detected by PCR in 50 of 50 DCA specimens (Figure 3). Cases were separated into high and low categories according to *C. pneumoniae* DNA copy numbers. The proportion of patients in the high copy-number category did not differ significantly between the SAP and ACS groups (12/25 [48%] vs 10/25 [40%], *P* = .78), or between patients with subsequent post-DCA restenosis and those without (5/12 [42%] vs 10/21 [48%], *P* = 1.00) (Table 2).

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**Table 1.** Clinical characteristics.

| CHARACTERISTICS | SAP (N=25) | ACS (N=25) | P VALUE* |
|-----------------|------------|------------|----------|
| Age (years, mean) | 63 ± 2 | 61 ± 2 | .53 |
| Male | 23 (92) | 19 (76) | .25 |
| Risk factor | | | |
| Hypertension | 12 (48) | 19 (76) | .08 |
| Hyperlipidemia | 12 (48) | 10 (40) | .88 |
| Hyperuricemia | 4 (16) | 8 (32) | .32 |
| Diabetes | 8 (32) | 6 (24) | .87 |
| Smoking | 6 (24) | 17 (68) | <.01 |
| Obesity | 5 (20) | 6 (24) | >.99 |
| Family history | 3 (12) | 4 (16) | >.99 |
| Culprit lesion | | | |
| LAD/LCX/LMT/RCA | 17/1/1/6 (68/4/4/24) | 24/1/0/0 (96/4/0/0) | |
| Medications | | | |
| Aspirins | 24 (96) | 16 (64) | .01 |
| Statins | 11 (44) | 5 (20) | .13 |

Data are expressed as the mean ± standard error of the mean or number (%). Abbreviations: ACS, acute coronary syndrome; LADCA, left anterior descending coronary artery; LCX, left circumflex artery; LMT, left main trunk; RCA, right coronary artery; SAP, stable angina pectoris.

*Welch t-test for numerical variables and Fisher’s exact test for categorical variables.

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**Figure 1.** Representative microphotographs of immunohistochemical staining for *C. pneumoniae*. Some smooth muscles (A) and many macrophages (B) in a coronary atherosclerotic plaque stained positive with anti-*C. pneumoniae* antibodies. Original magnification ×400.
In this study, *C. pneumoniae* was detected in all 50 DCA samples of coronary atherosclerotic lesions by both IHC and PCR. The reported detection rates of *C. pneumoniae* in coronary atherosclerotic lesions have been variable. In autopsy studies, 3% to 38% of “mild atherosclerosis” and 14% to 86% of “severe atherosclerosis” have been found to harbor *C. pneumoniae* using IHC or PCR. The higher detection rate in our study may be partially due to differences in detection methods, but more likely related to our use of DCA samples: DCA is a procedure to shave off culprit atherosclerotic lesions that are causing ischemic symptoms and these lesions can be considered to represent the final stage of atherosclerosis progression. That the detection rate of *C. pneumoniae* is highest in the most advanced atherosclerotic lesions is consistent with previous reports.

Nowadays, the association between *C. pneumoniae* and atherosclerosis is not as hotly debated as it once was, but the connection has not been ruled out. The failures of antibiotic therapy targeting *C. pneumoniae* to prevent cardiovascular accidents in patients with established coronary atherosclerotic...
In summary, our study revealed that *C. pneumoniae* is highly prevalent in coronary atherosclerotic plaques of patients who have severe enough disease to undergo DCA. The extent of *C. pneumoniae* infection in coronary atherosclerotic plaques was not associated with plaque instability or post-DCA restenosis.

**Author Contributions**

TO drafted the manuscript. Y Asada and KH conceived the study. TO, AY, and KH performed histopathological analysis. Y Azuma performed molecular analysis. KN and YS collected clinical data and specimens. All authors revised the manuscript.

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