Rapid Detection of *Chlamydia pneumoniae* DNA in Peripheral Blood Mononuclear Cells of Coronary Artery Disease Patients by Real-Time Fluorescence PCR

WATTANA LEOWATTANA, M.D.*, NITHI MAHANONDA, M.D.**,
SASIKANT POKUM, B.Sc.*, NARAVAT POUNGVARIN, M.D.*

Abstract

Several recent reports including serological, pathological and animal studies have associated *Chlamydia pneumoniae* with coronary artery disease (CAD). In order to establish whether chronic *C. pneumoniae* infection is linked to coronary artery disease, clinical intervention trials may be needed. However, to detect eligible patients with persistent infection, a reliable diagnostic marker must be developed for identifying cases and assessing efficacy of antichlamydial therapy. Moreover, the prevalence of circulating *C. pneumoniae* DNA in CAD patients varied widely from previous reports. A real-time PCR has been established by using HL-1 and HR-1 primer to amplify 437 base pairs product. Confirmation of the product was performed on LightCycler by melting curve analysis of detection probes labeled with LC-Red705. Ninety-five angiographically confirmed CAD patients and 104 normal, healthy volunteers were recruited. The mononuclear cell layer was separated from collected blood and rapid, single step real-time PCR was used to detect *C. pneumoniae* DNA. *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMC) was found in 17 per cent of 95 CAD patients and 1 per cent of 104 normal healthy volunteers (odds ratio 20.86, 95% confidence interval 2.71 – 160.67, $p < 0.0001$). There was no association between *C. pneumoniae* DNA in PBMC and serological status. The rapid, real-time PCR showed a clear-cut result between positive and negative cases. PBMC-based real-time PCR may be a useful tool for identifying subjects carrying *C. pneumoniae* in the circulation or in the vascular wall as well. It will be a specific indicator of current infection and will be used as a marker for assessing the microbiological efficacy of antichlamydial therapy in clinical intervention trials.

Key word : *Chlamydia pneumoniae* DNA, Peripheral Blood Mononuclear Cells and Real-Time Fluorescence PCR

**LEOWATTANA W, MAHANONDA N,
POKUM S, POUNGVARIN N
J Med Assoc Thai 2001; 84 (Suppl 3): S658-S668**

* Division of Clinical Chemistry, Department of Clinical Pathology,
** Her Majesty Cardiac Center, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

The serological association between *Chlamydia pneumoniae* (*C. pneumoniae*) and coronary artery disease (CAD) was first reported in 1988 and has subsequently been confirmed by numerous serological studies[1-5]. It has become mandatory to define a method for the identification of chronic *C. pneumoniae* carriers. Traditionally, a microimmunofluorescence (MIF) test has been considered to be a diagnostic "gold standard", but it cannot be used for identifying subjects with an ongoing infection. The direct techniques that determine the presence of *C. pneumoniae* DNA are therefore, preferable and rely mainly upon PCR techniques[6-9]. Although *C. pneumoniae* DNA has been repeatedly identified in atheromatous plaques, this method has little use as a large-scale clinical diagnosis of chronic *C. pneumoniae* infection because of the difficulty in obtaining plaque tissue from every patient. Recently, the detection of circulating *C. pneumoniae* DNA has been reported, but the estimation of prevalence has varied widely between 2.9 per cent to 46 per cent for normal subjects and 8.8 per cent to 59 per cent for heart disease patients[10-17]. The differences in prevalence of *C. pneumoniae* DNA the in peripheral blood mononuclear cells (PBMC) may be due to population or methodological differences. However, the techniques for detection of *C. pneumoniae* DNA in the circulation may be a potential marker of chronic *C. pneumoniae* infection. We present here a rapid single-step method for detection of *C. pneumoniae* DNA in PBMC of coronary artery disease (CAD). This method used rapid-cycle PCR and subsequent analysis with resonance energy transfer probes on a thermal cycler with real-time fluorescence monitoring. After the completion of the amplification process, the fluorescence signal was used to analyze the melting curves of fluorophore-labeled hybridization probe.

MATERIAL AND METHOD

Samples

Between September 2000 and March 2001, 95 consecutive CAD patients (58 males; and 37 females mean age, 61.9 ± 9.5 years; age range, 45-75 years) who underwent coronary angiography were enrolled. One hundred and four normal healthy volunteers (50 males; and 54 females mean age, 48.9 ± 11.2 years; age range 42-72 years) were enrolled as the control group. Informed consent was obtained from all participants. The local ethics committee approved this study. Venous blood was collected by percutaneous venepuncture in EDTA-treated tubes and layered over Ficoll-Paque (Pharmacia Biotech). Cells were centrifuged at 2000 rpm for 15 minutes, and the PBMC band was aspirated, washed twice and suspended in PBS prior to storage at -70°C pending for PCR analysis.

C. pneumoniae DNA detection using fluorescence resonance energy transfer

Fluorescence monitoring using hybridization probes is based on the concept that a fluorescence signal is generated if fluorescence resonance energy transfer (FRET) occurs between two adjacent fluorophores. The first hybridization probe, which is labeled with fluorescein as donor fluorophore on it 3′ end, can hybridize in close proximity to a second hybridization probe that is labeled with the acceptor fluorophore LightCycler™ Red 705 at its 5′ end and is blocked from extension at its 3′ terminus. The fluorescein is excited by an LED light source and transfers energy to the acceptor fluorophore. The acceptor fluorophore then emits light of a longer wavelength that can be measured with a photodiode. This detection strategy allows monitoring of the amplification process on a per-cycle basis because the intensity of the FRET signal depends on the amount of specific PCR product generated. Continuous monitoring of the fluorescence as the temperature is raised from annealing to denaturation produces a sharp decrease in fluorescence when the detection probe dissociates from template.

Primers and fluorogenic probes

The primer 5′-GTT GTT CAT GAA GGC CTA CT-3′ (HL-1) and the primer 5′-TGC ATA ACC TAC GGT GTG TT-3′ (HR-1) were used to amplify a 437-bp fragment of *C. pneumoniae* DNA. The amplification primers were synthesized by standard phosphoramidite chemistry (Biosyn, USA). The detection probe was a 21-mer oligonucleotide, labeled at the 3' end with fluorescein. The sequence 5′-GGC TGG ACG ACA CGG AAA TAA-(fluorescein)- 3′ (HFL-1) is complementary to the leading strand of the *C. pneumoniae* amplicon. The anchor probe 5′-(red 705)-TTG TTT CCA AAA TCG TTC-(phosphate)-3′ (HFL-2) was a 18-mer labeled with LightCycle Red 705 at its 5′ end and modified at the 3′ end by phosphorylation to block extension. The anchor probe binds at a distance 5

bases 3' from the detection probe. Both fluorophore-labeled probes were synthesized and purified by reversed-phase HPLC (GENSET, Singapore).

Fluorescence protocol

The analysis was carried out on a LightCycler (Roche Diagnostics, Switzerland). PCR was performed by rapid cycling in a reaction volume 20 µL with 0.5 µmol/L each primer, 0.2 µmol/L anchor and detection probes and 50 ng of extracted DNA from PBMC of CAD patients, infected HEp2 cells with *C. pneumoniae* as positive control or distilled water as negative control. The LightCycler DNA master hybridization probes buffer was provided as a 10-fold solution containing nucleotides, *Thermus aquaticus (Taq)* DNA polymerase, and 10 mmol/L Mg^{2+}. The final Mg^{2+} concentration in the reaction mixture was adjusted to 4 mmol/L. The samples were loaded into glass capillary cuvettes and centrifuged to place the sample at the capillary tip before capping. After an initial denaturation step at 95°C for 10 m, amplification was performed using 50 cycles of denaturation (95°C for 10 s), annealing (53°C for 10 s) and extension (72°C for 15 s). Fluorescence was measured at the end of the annealing period of each cycle to monitor amplification. After amplification was complete, a final melting curve was recorded by cooling the reaction mixture to 40°C at 20°C/s and then slowly heated it to 85°C at 0.05°C/s. Fluorescence was measured continuously during the slow temperature ramp to monitor the dissociation of the fluorescein-labeled detection probe. The fluorescence signal (F) was plotted in real time against temperature (T) to produce melting curves for each sample (F *vs* T). Melting curves were then converted to derivative melting curves by plotting the negative derivative of the fluorescence with respect to temperature against temperature (-(dF/dT) *vs* T). The total process took around 45 min with no post PCR procedures.

Serology for *C. pneumoniae*

C. pneumoniae serology data were obtained by ELISA, anti-*C. pneumoniae* IgG and IgA were measured by SeroCP IgG, IgA (Savyon Diagnostics, Israel) respectively. Anti-*C. pneumoniae* IgG or IgA seropositivity was defined as a cutoff index of 1.1 or greater, where the index was obtained by dividing the specimen optical density by twice the mean of three negative controls in accordance with the manufacturer's product insert.

Lipid profiles determinations

Serum total cholesterol and triglycerides were determined by an automated Hitachi 717 (Roche Diagnostics, Switzerland). Serum high-density lipoprotein cholesterol and low-density lipoprotein cholesterol were measured by direct method on Hitachi 717 according to manufacturer's protocols.

Statistical analysis

Patients' and controls' demographic data, baseline variables and lipid profiles were compared by student *t*-test, χ^2 and Mann-Whitney *U* test as appropriate (SPSS for Windows, SPSS Inc, USA). Odd ratio and 95 per cent confident interval were calculated by EpiInfo (CDC, USA). A p value of < 0.05, two-tailed, was considered statistically significant.

RESULTS

Fifty cycles of amplification were performed with PBMC DNA from CAD patients, *C. pneumoniae* DNA from HEp2 cells (positive control) or distilled water (negative control), using the FRET detection system outlined in Fig. 1. When fluorescence was measured at the end of each annealing phase, the fluorescence signal increased as product accumulated and the signal appeared above the background after 24 cycles as shown in Fig. 2. The process of hybridization and melting of the detection probe to the target was monitored by melting curve analysis. Melting of the positive control *C. pneumoniae* DNA produced a rapid decrease in fluorescence at 61 - 62°C contrary to negative control produced flat curve (Fig. 3). The PBMC DNA from CAD patients who carry *C. pneumoniae* DNA produced the same melting peak with positive control. To evaluate the reliability of real-time fluorescence PCR technique, the PCR products were run electrophoresis with 2 per cent agarose gel and stained with ethidium bromide. The generated fragments on the LightCycler for positive *C. pneumoniae* were 437 bp that was the same as the expected size (Fig. 4).

Table 1 shows the age, sex distribution, major risk factors and lipid profiles of CAD patients and normal healthy volunteers. These two groups showed a significant difference in mean age, hypertension, diabetes mellitus, hyperlipidemia and serum HDL-C concentration. Sixteen of 95 PBMC DNA (16.84%) from CAD patients were positive by fluorescence real-time PCR. When the PBMC fractions from 104 normal healthy volunteers were analyzed,

Fig. 1. Relative orientation of the fluorophore-labeled anchor and detection probes.The detection probe spanning the cloned *PstI* at position 313 - 346 upstream from the start of transcription is labeled at the 3′ end with fluorescein (FITC). The anchor probe, labeled with LightCycler Red705 (LCRed 705) at the 5′ end, is phosphorylated at its 3′ end to block extension and bind at a distance five nucleotides 3′ from the detection probe.

1 (0.96%) was repeatedly PCR positive with an odds ratio (OR) equal to 20.86 and 95 per cent confidence interval (CI) between 2.71 and 160.67, p value < 0.0001 (Table 2). The prevalence of positive anti-*C. pneumoniae* IgG was significantly higher in CAD patients than in healthy volunteers giving OR equal to 3.60 and 95 per cent CI between 1.95 and 6.64, p value < 0.0001. Regarding the prevalence of positive anti-*C. pneumoniae* IgA, the two groups were not significantly different. The detection rates of *C. pneumoniae* DNA in PBMC according to serological status are shown in Table 3. Anti-*C. pneumoniae* IgG results were positive for 6 of 17 subjects (35%) with *C. pneumoniae* DNA- positive PBMC, *versus* 65 of 180 subjects (36%) who were DNA negative (OR = 1.02, 95%CI = 0.39 - 2.71, p value = 0.8). The positive rates of anti-*C. pneumoniae* IgA were the same as anti-*C. pneumoniae* IgG.

DISCUSSION

Increasing evidence indicates that infections, either bacterial or viral, play an important role in the origin of atherosclerosis through inflammation and endothelial damage. *C. pneumoniae* is now widely accepted as being a common respiratory pathogen of human. It has been strongly linked to atherosclerotic heart disease by a wide range of detection procedures. The monocyte is a likely cell for dissemination of *C. pneumoniae* to non-respiratory sites especially coronary arteries. In this study the authors presented a rapid, real-time PCR method, which has no need for post-PCR procedures in detection of *C. pneumoniae* DNA from PBMC. The prevalence of *C. pneumoniae* DNA from 95 CAD patients was 17 per cent, which is similar to the results of four studies from United Kingdom, Canada and Germany[14-17]. The much higher prevalence of PBMC *C. pneumoniae* DNA reported from Sweden (59.4% of 101 CAD patients) and USA (36% of 28 CAD patients) might reflect population or methodology differences[10,13]. The finding that positive serology results were not associated with circulating *C. pneumoniae* DNA was similar to all of the previous studies[18-20]. These studies may explain why anti-*C. pneumoniae* IgG or IgA did not predict cardiovascular events or chronic carrier cases in several prospective studies.

The authors examined the reproducibility of PCR results. The amplification product was 437 bp of *C. pneumoniae* DNA and confirmed by hybridization with a specific oligonucleotide probe. This

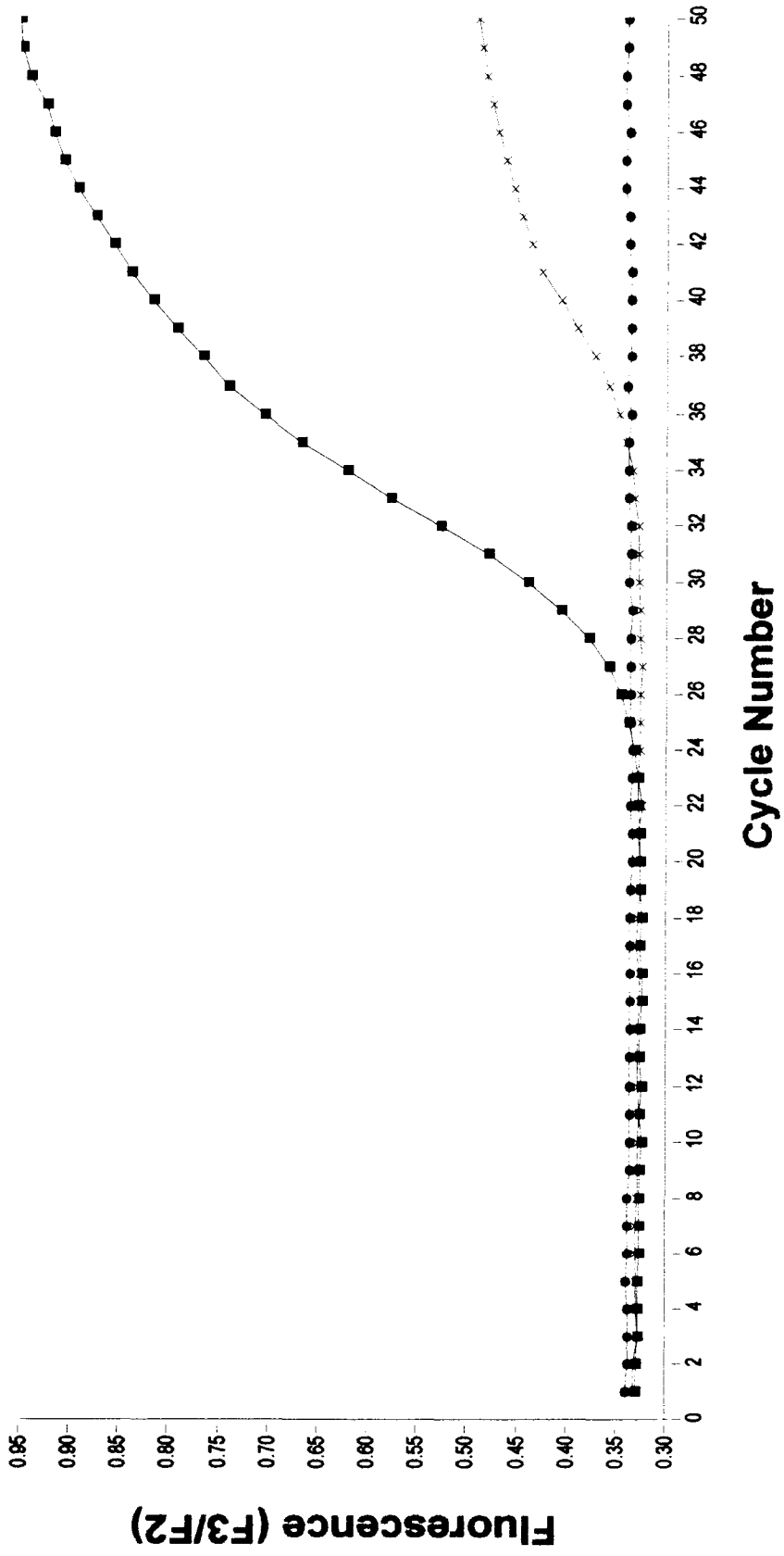

Fig. 2. Intensity of the fluorescence signal (F) *vs* cycle number. A 437 bp fragment of *C. pneumoniae* DNA was amplified: positive control (■—■—■), positive case (✱—✱—✱). Amplification of a no-template (distilled water) control (●—●—●) was also attempted. The fluorescence signal was acquired once each cycle at the end of the annealing period.

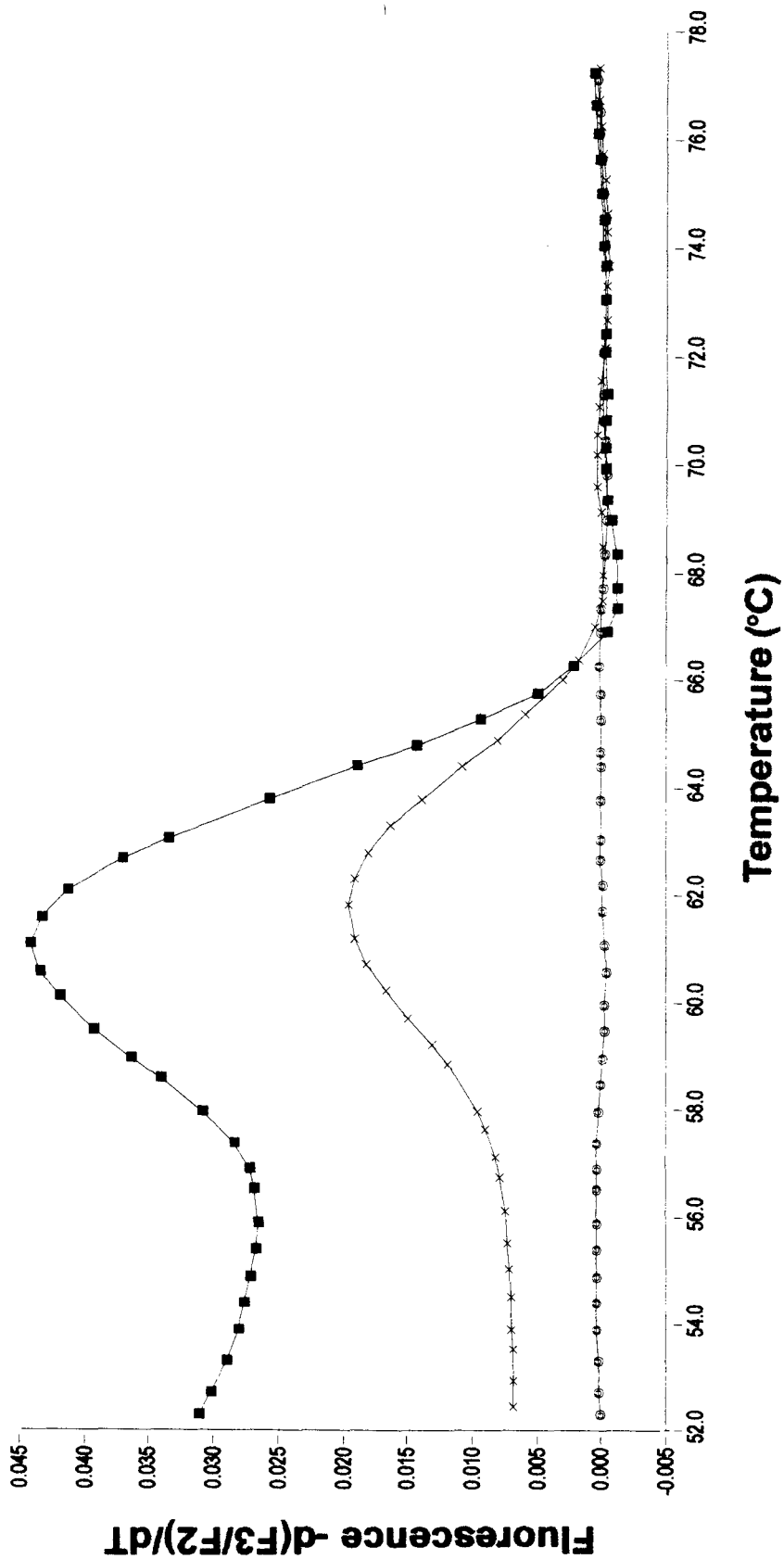

Fig. 3. Positive control (■—■—■), positive case (✱—✱—✱) and negative control (●—●—●) with a specific fluorescence probe by derivative melting curve plots.

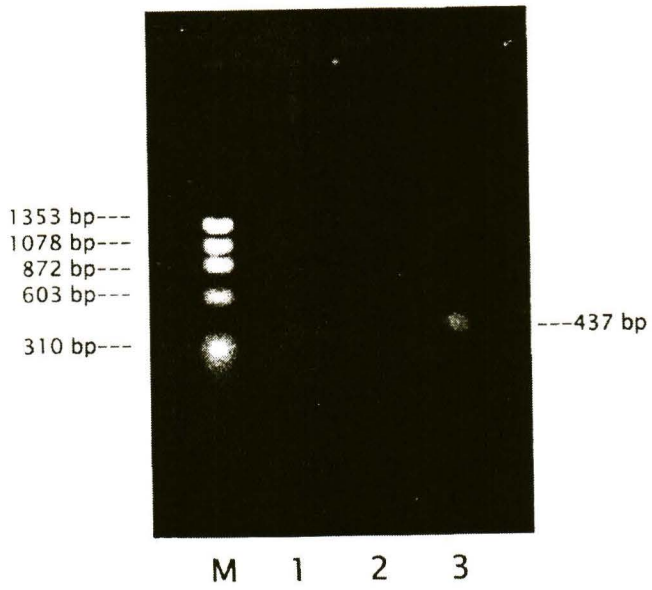

Fig. 4. Agarose gel electrophoresis analysis of PCR products from LightCycler. Gels were stained with ethidium bromide and photographed under UV light at 305 nm. PCR assay products are as follows: lane 1, primers; lane 2, distilled water; lane 3, positive case. M, molecular size standards.

Table 1. Demographic data, major risk factors and lipid profiles of 95 CAD patients and 104 normal healthy volunteers.

	CAD patients (n=95)	%	Healthy volunteers (n=104)	%	p value
Age, years	61.9 ± 9.5		48.9 ± 11.2		<0.0001
Male	70	70	65	65	NS
Hypertension	55	57	22	21	<0.0001
Diabetes mellitus	35	37	15	14	0.0002
Smoking	34	36	24	23	NS
Hyperlipidemia	65	68	53	51	0.0183
Family history of CAD	26	27	17	16	NS
TC, mmol/L	5.5 ± 1.3		5.7 ± 1.0		NS
TG, mmol/L	1.9 ± 1.2		1.8 ± 1.0		NS
HDL-C, mmol/L	1.0 ± 0.3		1.3 ± 0.4		<0.0001
LDL-C, mmol/L	3.5 ± 1.3		3.5 ± 0.9		NS

TC = total cholesterol, TG = triglycerides, HDL-C = high density lipoprotein cholesterol, LDL-C = low density lipoprotein cholesterol, NS = not significant

rapid, single step, real-time PCR method performed on peripheral blood may be a good marker for identification of subjects carrying *C. pneumoniae* in mononuclear cells, although it does not prove the presence of viable *Chlamydia*. Recently, Bodetti TJ, et al reported the correlation between PCR result and antigen detection by antibody staining methods, which support the hypothesis that, these PBMCs are truly infected and the PCR-positive status does not simply represent remnant pieces of nucleic acid

Table 2. Detection of *C. pneumoniae* DNA in PBMC and serological status in CAD patients and healthy volunteers.

	CAD patients (n=95)	%	Healthy volunteers (n=104)	%	OR (95%CI)	p value
Cp DNA in PBMC	16	17	1	1	20.86 (2.71-160.67)	<0.0001
Anti-Cp IgG	48	51	23	22	3.60 (1.95-6.64)	<0.0001
Anti-Cp IgA	37	39	38	37	1.67 (0.60-1.91)	NS

Cp = *Chlamydia pneumoniae*, NS = not significant.

Table 3. Association of *C. pneumoniae* DNA in PBMC and serological status.

	No. of positive/total					
	DNA negative	%	DNA positive	%	OR (95%CI)	p value
Anti-Cp IgG	65/182	36	6/17	35	1.02 (0.39-2.71)	NS
Anti-Cp IgA	69/182	38	6/17	35	1.09 (0.41-2.87)	NS

Cp = *Chlamydia pneumoniae*, NS = not significant

resulting from the degradative activity of the monocytes[12]. In this regard, Smieja M, et al proposed regarding the results of positive PCR, which increases after coronary angiography, that *C. pneumoniae* in macrophages within the atherosclerotic plaque would seed the bloodstream during and after the angiography,due to the disruption of the endothelium[17]. They also showed no association between positive *C. pneumoniae* and subsequent revascularization or other clinical events after 6 month's follow-up.

An association between circulating *C. pneumoniae* DNA and CAD does not necessarily imply causation. However, a small secondary prevention trial has reported that azithromycin reduces the incidence of further adverse coronary events following myocardial infarction[21]. A second study also reported significant benefits after thirty-day's follow-up but not after six months[22,23]. The third study also found no benefit from azithromycin treatment on a larger scale trial[24]. Further trials are underway in many countries, with longer periods of antibiotic treatment. One trial will prescribe antibiotics for periods of up to one year without evidence of prior infection[25]. To assess physicians' knowledge, attitudes, and prescribing behaviors with regard to the association between *C. pneumoniae* and cardiovascular disease, Gimenez-Sanchez F, et al surveyed 2,481 physicians in the USA and nationwide network in 1999[26]. They reported that 85 per cent of physicians knew of this association; and 4 per cent of them had treated or recommended treating cardiovascular diseases with antimicrobial agents. Antimicrobials are only likely to benefit those who are currently infected with *C. pneumoniae* and appropriate prescribing should be given to subjects in whom there is good evidence to confirm current *C. pneumoniae* infection. Moreover, Gieffers J, et al concluded that *C. pneumoniae* infection in circulating human monocytes is refractory to azithromycin treatment[27]. This notion is supported by recent data from ongoing treatment trials[28-30].

A direct detection method for *C. pneumoniae* that uses a peripheral blood sample, therefore, appears to be a promising new diagnostic tool. Crucial steps that must be carefully optimized for reliable results include separation of PBMC and the choice of DNA extraction method. Furthermore, the use of an optimized, sufficiently controlled PCR method is of the utmost importance for the sensitive detection of *C. pneumoniae* in these types of specimens. It would also be useful to have access to quantitative assays for determining the chlamydial load in carrier cases, especially for monitoring the effect therapy in treatment trials. Quantification of *C. pneumoniae* DNA in circulating white blood cells may be possible by use of a real-time PCR as presented in this study.

In conclusion, the real-time PCR or other amplification techniques that can detect *C. pneumoniae* in circulating white blood cells may be of great value for identifying *C. pneumoniae* carriers and for monitoring antichlamydial therapy. These real-time or nested PCR techniques, which are easily done with available blood specimens, may be used as a valid surrogate marker for identification of persons with persistent *C. pneumoniae* infection, and strengthen the diagnostic tools in intervention trials, especially if rapid, reliable and quantitative assays are used.

ACKNOWLEDGEMENT

This study was supported in part by a grant from China Medical Board, Faculty of Medicine Siriraj Hospital, Mahidol University.

(Received for publication on September 21, 2001)

REFERENCES

1. Saikku P, Leinonen M, Mattila K, et al. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. Lancet 1988; 2: 983-6.
2. Saikku P. Epidemiologic association of *Chlamydia pneumoniae* and atherosclerosis: The initial serologic observation and more. J Infect Dis 2000; 181 (Suppl 3): S411-3.
3. Danesh J, Collins R, Peto R. Chronic infections and coronary heart disease: Is there a link? Lancet 1997; 350: 430-6.
4. Danesh J, Whincup P, Walker M, et al. *Chlamydia pneumoniae* IgG titres and coronary heart disease: Prospective study and meta-analysis. BMJ 2000; 321: 208-13.
5. Leowattana W, Mahanonda N, Bhuripunyo K, Leelarasamee A, Pokum S, Suwimol B. The prevalence of *Chlamydia pneumoniae* antibodies in Thai patients with coronary artery disease. J Med Assoc Thai 1999; 82: 792-7.
6. Farsak B, Yildirir A, Akyon Y, et al. Detection of *chlamydia pneumoniae* and *helicobacter pylori* DNA in human atherosclerotic plaques by PCR. J Clin Microbiol 2000; 38: 4408-11.
7. Berger M, Schroder B, Daeschlein G, et al. *Chlamydia pneumoniae* DNA in non-coronary atherosclerotic plaques and circulating leukocytes. J Lab Clin Med 2000; 136: 194-200.
8. Esposito G, Blasi F, Allegra L, et al. Demonstration of viable Chlamydia pneumoniae in atherosclerotic plaques of carotid arteries by reverse transcriptase polymerase chain reaction. Ann Vasc Surg 1999; 13: 421-5.
9. Blasi F, Denti F, Erba M, et al. Detection of *Chlamydia pneumoniae* but not *Helicobacter pylori* in atherosclerotic plaques of aortic aneurysms. J Clin Microbiol 1996; 34: 2766-9.
10. Boman J, Soderberg S, Forsberg J, et al. High prevalence of *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells in patients with cardiovascular disease and in middle-aged blood donors. J Infect Dis 1998; 178: 274-7.
11. Blasi F, Boman J, Esposito G, et al. *Chlamydia pneumoniae* DNA detection in peripheral blood mononuclear cells is predictive of vascular infection. J Infect Dis 1999; 180: 2074-6.
12. Bodetti TJ, Timms P. Detection of *Chlamydia pneumoniae* DNA and antigen in the circulating mononuclear cell fractions of humans and koalas. Infect Immun 2000; 68: 2744-7.
13. Kaul R, Uphoff J, Wiedeman J, Yadlapalli S, Wenman WM. Detection of *Chlamydia pneumoniae* DNA in CD3+ lymphocytes from healthy blood donors and patients with coronary artery disease. Circulation 2000; 102: 2341-6.
14. Mahony JB, Chong S, Coombes BK, Smieja M, Petrich A. Analytical sensitivity, reproducibility of results, and clinical performance of five PCR assays for detecting *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells. J Clin Microbiol 2000; 38: 2622-7.
15. Maass M, Jahn J, Gieffers J, Dalhoff K, Katus HA, Solbach W. Detection of *Chlamydia pneumoniae* within peripheral blood monocytes of patients with unstable angina or myocardial infarction. J Infect Dis 2000; 181 (Suppl 3): S449-51.
16. Wong YK, Dawkins KD, Ward ME. Circulating *Chlamydia pneumoniae* DNA as a predictor of coronary artery disease. J Am Coll Cardiol 1999; 34: 1435-9.
17. Smieja M, Chong S, Natarajan M, Petrich A, Rainen L, Mahony JB. Circulating nucleic acids of *Chlamydia pneumoniae* and cytomegalovirus in patients undergoing coronary angiography. J Clin

Microbiol 2001; 39: 596-600.
18. Ridker PM, Hennekens CH, Buring JE, Kundsin R, Shih J. Baseline IgG antibody titers to *Chlamydia pneumoniae*, *Helicobacter pylori*, herpes simplex virus, and cytomegalovirus and the risk for cardiovascular disease in women. Ann Intern Med 1999; 131: 573-7.
19. Ridker PM, Kundsin RB, Stampfer MJ, Poulin S, Hennekens CH. Prospective study of *Chlamydia pneumoniae* IgG seropositivity and risks of future myocardial infarction. Circulation 1999; 99: 1161-4.
20. Nieto FJ, Folsom AR, Sorlie PD, Grayston JT, Wang SP, Chambless LE. *Chlamydia pneumoniae* infection and incident coronary heart disease: The Atherosclerosis Risk in Communities Study. Am J Epidemiol 1999; 150: 149-56.
21. Gupta S, Leatham EW, Carrington D, Mendall MA, Kaski JC, Camm AJ. Elevated *Chlamydia pneumoniae* antibodies, cardiovascular events, and azithromycin in male survivors of myocardial infarction. Circulation 1997; 96: 404-7.
22. Gurfinkel E, Bozovich G, Daroca A, Beck E, Mautner B. Randomised trial of roxithromycin in non-Q-wave coronary syndromes: ROXIS Pilot Study. ROXIS Study Group. Lancet 1997; 350: 404-7.
23. Gurfinkel E, Bozovich G, Beck E, Testa E, Livellara B, Mautner B. Treatment with the antibiotic roxithromycin in patients with acute non-Q-wave coronary syndromes. The final report of the ROXIS Study. Eur Heart J 1999; 20: 121-7.
24. Anderson JL, Muhlestein JB, Carlquist J, et al. Randomized secondary prevention trial of azithromycin in patients with coronary artery disease and serological evidence for *Chlamydia pneumoniae* infection: The Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with Chlamydia (ACADEMIC) study. Circulation 1999; 99: 1540-7.
25. Grayston JT. Antibiotic treatment trials for secondary prevention of coronary artery disease events. Circulation 1999; 99: 1538-9.
26. Gimenez-Sanchez F, Butler JC, Jernigan DB, et al. Treating Cardiovascular Disease with antimicrobial agents: A survey of knowledge, attitudes, and practices among physicians in the United States. Clin Infect Dis 2001; 33: 171-6.
27. Gieffers J, Fullgraf H, Jahn J, et al. *Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic treatment. Circulation 2001; 103: 351-6.
28. Jackson LA, Smith NL, Heckbert SR, Grayston JT, Siscovick DS, Psaty BM. Lack of association between first myocardial infarction and past use of erythromycin, tetracycline, or doxycycline. Emerg Infect Dis 1999; 5: 281-4.
29. Muhlestein JB, Anderson JL, Carlquist JF, et al. Randomized secondary prevention trial of azithromycin in patients with coronary artery disease: primary clinical results of the ACADEMIC study. Circulation 2000; 102: 1755-60.
30. Meier CR, Derby LE, Jick SS, Vasilakis C, Jick H. Antibiotics and risk of subsequent first-time acute myocardial infarction. JAMA 1999; 281: 427-31.

การตรวจหาดีเอ็นเอของเชื้อ *คลามิเดีย นิวโมนิอี* ในเม็ดเลือดขาวไหลเวียนอยู่ในกระแสเลือดของผู้ป่วยโรคหลอดเลือดหัวใจโดยวิธีเรียลไทม์ ฟลูออเรสเซ็น พีซีอาร์

วัฒนา เลี้ยววัฒนา, พ.บ., นิธิ มหานนท์, พ.บ.**,*
ศศิกานต์ โพธิ์คำ, วท.บ., นรวรรธน์ พวงวรินทร์, พ.บ.**

จากการรายงานของนักวิจัยจำนวนมากถึงความสัมพันธ์ของภาวะติดเชื้อ *คลามิเดีย นิวโมนิอี* กับโรคหลอดเลือดหัวใจทั้งในด้านภูมิคุ้มกัน พยาธิวิทยา รวมทั้งการศึกษาในสัตว์ทดลอง พบว่ามีความเป็นไปได้สูงมากที่เชื้อ *คลามิเดีย นิวโมนิอี* จะมีส่วนในการเกิดโรคดังกล่าว อย่างไรก็ตามยังต้องการการพิสูจน์ที่ชัดเจนโดยการศึกษาทางคลินิกโดยการให้ยาปฏิชีวนะแก่ผู้ป่วยที่สงสัยว่าจะมีการติดเชื้อ *คลามิเดีย นิวโมนิอี* แบบเรื้อรัง แต่ปัจจุบันยังไม่สามารถหาวิธีการตรวจยืนยันการติดเชื้อ *คลามิเดีย นิวโมนิอี* เรื้อรัง ที่ถูกต้องและแม่นยำได้ คณะผู้วิจัยจึงได้คิดค้นและพัฒนาวิธีการตรวจยืนยันด้วยการใช้ เรียลไทม์ พีซีอาร์ เทคนิกโดยใช้ HL-1 และ HR-1 เป็น primer ในการเพิ่มจำนวนดีเอ็นเอของเชื้อ *คลามิเดีย นิวโมนิอี* ที่อยู่ในเม็ดเลือดขาวส่วนที่ไหลเวียนโดยมีขนาดของผลผลิตสายดีเอ็นเอเท่ากับ 437 คู่เบส และยืนยันโดยการตรวจดู melting curve ของ hybridization probe ที่ติดฉลากด้วยสี LC-Red705 ประชากรที่ศึกษาประกอบด้วยผู้ป่วยโรคหลอดเลือดหัวใจที่ตรวจยืนยันโดยการตรวจสวนหลอดเลือดหัวใจจำนวน 95 รายเปรียบเทียบกับอาสาสมัครสุขภาพดีจำนวน 104 รายพบ ดีเอ็นเอของเชื้อ *คลามิเดีย นิวโมนิอี* ในเม็ดเลือดขาว 17% ของผู้ป่วยโรคหลอดเลือดหัวใจ ซึ่งมากกว่าในอาสาสมัครสุขภาพดีที่พบเพียง 1% เป็นอย่างมาก (odd ratios 20.86, 95%CI 2.71 – 160.67, p < 0.0001) โดยไม่พบความสัมพันธ์ระหว่างดีเอ็นเอของเชื้อ *คลามิเดีย นิวโมนิอี* ในเม็ดเลือดขาวและภูมิต้านทานที่ตรวจพบ แสดงให้เห็นว่าการตรวจหาดีเอ็นเอของเชื้อ *คลามิเดีย นิวโมนิอี* ด้วยวิธีดังกล่าวสามารถแยกผู้ป่วยที่มีการติดเชื้อ *คลามิเดีย นิวโมนิอี* แบบเรื้อรังออกจากคนปกติได้อย่างชัดเจน นอกเหนือจากนั้นวิธีการตรวจยังทำได้ง่าย รวดเร็วและค่าใช้จ่ายไม่สูงมาก จึงสามารถนำวิธีการตรวจนี้มาใช้ในการค้นหาผู้ป่วยโรคหลอดเลือดหัวใจที่ติดเชื้อ *คลามิเดีย นิวโมนิอี* แบบเรื้อรังและต้องการการรักษาด้วยยาปฏิชีวนะได้โดยไม่ยากนัก

คำสำคัญ : *คลามิเดีย นิวโมนิอี* ดีเอ็นเอ, เม็ดเลือดขาวไหลเวียนอยู่ในกระแสเลือด, เรียลไทม์ ฟลูออเรสเซ็น พีซีอาร์

วัฒนา เลี้ยววัฒนา, นิธิ มหานนท์,
ศศิกานต์ โพธิ์คำ, นรวรรธน์ พวงวรินทร์
จดหมายเหตุทางแพทย์ ฯ 2544; 84 (ฉบับพิเศษ 3): S658-S668

* สาขาวิชาเคมีคลินิก, ภาควิชาพยาธิวิทยาคลินิก,
** ศูนย์โรคหัวใจสมเด็จพระบรมราชินีนาถ, คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กรุงเทพ ฯ 10700