Reliability of Nested PCR for the Detection of *Chlamydia pneumoniae* in Carotid Artery Atherosclerosis

To the Editor:

We recently read the article by Prager et al, who reported finding *Chlamydia pneumoniae* DNA in more than 80% of atherosclerotic carotid artery plaques of 46 patients suffering from symptomatic carotid artery stenosis. Moreover, about 85% of patients also had detectable *C pneumoniae* DNA in their circulating white blood cells. In contrast, the saphenous veins of these subjects revealed a surprisingly low *C pneumoniae* DNA positivity ratio of 6.5%.

We wonder if the authors and/or any of the referees who have reviewed this article were familiar with the problems and limitations of PCR testing for *C pneumoniae*, including DNA extraction methods and PCR technology in general (and know the differences between this and doing a serum cholesterol or CRP level) and with the detection of *C pneumoniae* DNA in atherosclerotic tissue in particular. *C pneumoniae* PCR is not standardized, all the assays reported in the literature are in-house tests, and most have not been validated compared with culture or even other PCR methods. Unfortunately, there is evidence of major problems with both inter- and intralaboratory reproducibility.\(^2\)\(^-\)\(^4\) Much of the variation in the prevalence of *C pneumoniae* DNA detected in human atheroma specimens reported in the literature (0% to 100%) is probably due to variation in methods. Also, several studies have failed to find an association between detection of *C pneumoniae* DNA in atheromomas and peripheral blood mononuclear cells.\(^5\)\(^-\)\(^7\) The conclusions of Prager et al are based on data that are probably biased by poor methodology.

Direct detection of *C pneumoniae* requires an exceptional level of know-how and expertise, considering not only cell culture and quantification but also detection of this pathogen by PCR, especially nested methods. To increase sensitivity and specificity, target DNA is amplified in a 2-step procedure using 2 different primer pairs. Unfortunately, for this purpose vials potentially containing highly positive concentrations of *Chlamydiae* DNA tend to be opened, which, without doubt, is associated with a high risk of contamination. How to control nested PCR technology to ensure reliable results is unknown. Moreover, it is more than doubtful if contamination can be controlled at all.\(^3\)

For the reasons outlined above, many study groups focusing on *C pneumoniae*-related research have stopped using nested PCR and switched to new, real-time-based PCR technologies.\(^5\)\(^-\)\(^7\)\(^,\)\(^8\)

Prager et al felt they had ruled out contamination as a reason for their high rate of positive samples because most of the venous samples (about 90%) were negative. This could only be assumed if venous samples were handled and/or tested simultaneously with the blood and atheroma specimens. In order to minimize the risk of false-positive results due to contamination of specimens with previous amplification products, positive controls, positive specimens, or exogenous sources, it is important to follow in detail the recommended guidelines published in a series of reviews focusing on the problems of detection of *C pneumoniae* by PCR.\(^9\)\(^-\)\(^11\)

The authors mention that their PCR’s “detected *C pneumoniae* at the required level of sensitivity.” However, they did not indicate what was the required level and, most important, what was the level of sensitivity of their assay in their laboratory, including the detection limits for purified DNA and mock-infected specimens. Critically important information would have been provided if all samples were also tested together with a similar number of known negative controls, which should include all PCR components except the target DNA, and run in parallel with the clinical samples throughout the whole procedure, beginning with DNA extraction to detection of amplification product. How many replicates were tested and what was considered to be a positive sample? How many negative controls were included per run? Was there any negative control present? Unfortunately, Prager et al did not provide any answers to these questions.

In addition, the authors mention that the application of 2 different PCR methods should ensure reliable results. However, the authors used a 16S rRNA-based PCR and confirmed findings by a 16S rRNA-based PCR. It would have had more impact if they had amplified a different *C pneumoniae*-specific gene, ie, the gene encoding for the major outer membrane protein (MOMP). If contamination has occurred during the first amplification it is more than likely that the second 16S rRNA PCR would have confirmed the contamination also. We wonder if authors have used any strategies to inactivate amplicon carryover (eg, using dUTP and N-uracil glycosylase) in the single-step PCR protocol. In addition, what was used as negative as well as positive controls at the various methodological levels? For example, the use of strongly positive controls should be avoided; the positive controls should consist of only low or very low DNA levels of *C pneumoniae* DNA. A number of studies as well as review articles\(^2\)\(^-\)\(^5\)\(^,\)\(^7\)\(^-\)\(^10\)\(^,\)\(^11\) have been published that have examined the reliability of PCR methodology for the detection of *C pneumoniae* in vascular tissue, none of which were cited by Prager et al.

We recently conducted 2 multicenter trials to compare various DNA extraction methods and PCR protocols for the detection of *C pneumoniae* DNA from endarterectomy specimens. Specimens were collected during the same period as those collected by Prager et al, from the same patient population, at the same hospital. In the first study,\(^2\) we sent a panel of identical atheroma specimens and controls to 9 laboratories in Europe and the United States; the reported positivity rates for detection of *C pneumoniae* DNA by PCR ranged from 0% to 60%, and there was no correlation between the detection rates and the sensitivity of the methods used. There was poor concordance between the different laboratories: only 25% agreed on 1 specimen.

It is noteworthy that the primers used by Prager et al were designed at one of the sites that participated in our study (Gaydos et al). However, Gaydos et al increased sensitivity by adding a *C pneumoniae*-specific probe and an enzyme-linked immunosorbent assay. Despite that, their method did not detect more than 1 *C pneumoniae*-DNA-positive coronary artery plaque out of a total of 56 specimens in their laboratory.\(^12\)

In our second study,\(^3\) 4 laboratories used a nested MOMP-based PCR on carotid artery plaques prepared by means of 3 different extraction methods under standardized conditions. Out of 240 PCR analyzed, only 5 (2%) were *C pneumoniae* DNA-positive. Even after exchange of DNA extracts between laboratories, the overall positivity rate did not exceed 5% (out of 720 analyses!), which was, nevertheless, lower than that within the negative controls (8%). Not one single positive result could be achieved when all atheroma extracts were reamplified by means of a 16S rRNA PCR followed by hybridization with a *C pneumoniae*-specific probe. Statistical analyses demonstrated...
that positive results could most likely be explained by ampiclon carry-over at nested PCR level as well as ampiclon introduction during DNA extraction.

Our study demonstrated that even experienced laboratories obviously had problems with contamination, which came to light only because of the study design. Compared with our study, the “low prevalence” of the venous samples reported by Prager et al could be what was found in our study to be the “background contamination rate” of 8%. Unfortunately, Prager et al do not give any details as what was done to control their nested PCR.

Hence, the results of prevalence studies like that of Prager et al, using nested PCR for detection of C pneumoniae, will always be more than questionable.

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Response

We have read with astonishment the letter by Apfalter et al in reference to our article in the December 2002 issue of Stroke.1 In their letter the authors raise serious doubts about our results. These doubts, however, are based exclusively on assumption and not on evidence, as should be good practice in science.

Apfalter et al suggested that our group and also the reviewers lack experience in the performance of the used techniques and in the interpretation of the respective data. If the authors of the letter had cared to check on the publication record of the people involved in our study, they would have realized that the authors of our study have extensive experience with PCR technology and thus are well aware of the differences between PCR and determination of serum cholesterol or CRP.2,7 We also are confident that Stroke, as a high-ranking and prestigious journal of the American Heart Association, carefully selects reviewers who are well experienced in the field of the respective study.

In order to address the points raised by Apfalter et al, we would like to emphasize that in fact venous samples, blood samples, and samples of the atherosclerotic plaque were processed and tested simultaneously and thus we still feel that the differences in the incidence of positive samples between leukocytes and plaques on the one hand and the healthy vein on the other hand most likely rules out contamination. We are of course well aware of the fact that, as in other assays or techniques, positive and negative controls have to be used also when performing PCR. In fact we have included 1 positive control and 1 negative control per run each consisting of 6 clinical samples (leukocytes, plaques and veins from 2 individual patients), respectively. Positive and negative controls were processed and treated exactly like the clinical samples and contained all the components used in the clinical samples. In all cases positive controls were positive whereas negative controls gave negative results. All samples were tested twice and gave identical results. Furthermore, we would like to mention that DNA from leukocytes was isolated following 2 different protocols. Both sets of DNA samples gave identical results.

We assume that the authors of this letter are not familiar with the fact that publications in Stroke are limited to a certain word count. In fact, when we submitted our manuscript, we were limited to 4500 words, and in order to meet this limit we had to reduce substantially the Subjects and Methods section and the reference list in our paper. We did so because we felt it more important and interesting to the reader to present and discuss our results than to extensively describe the methodological procedures.

We do not understand as to how using the gene encoding for the major outer membrane protein (MOMP) for nested PCR would have ruled out contamination. In fact, if the samples had been contaminated in the first run of normal PCR then contamination would have been also evident in the second run of nested PCR using a different gene. In addition, it is noteworthy that one of the authors of this letter by Apfalter et al used MOMP-based nested PCR and found 59% of samples of peripheral blood mononuclear cells (PBMC) obtained from patients with cardiovascular disease—a value that is not too different from the prevalence published in our article—and 46% of samples of PBMC obtained from healthy blood donors to be Chlamydia pneumoniae-positive.8

With respect to the multicenter trial mentioned by Apfalter et al, we would like to emphasize that out of 15 clinical samples tested in their study only 6 were obtained from carotid artery samples. We assume that the authors of this letter are not familiar with the fact that publications in Stroke are limited to a certain word count. In fact, when we submitted our manuscript, we were limited to 4500 words, and in order to meet this limit we had to reduce substantially the Subjects and Methods section and the reference list in our paper. We did so because we felt it more important and interesting to the reader to present and discuss our results than to extensively describe the methodological procedures.

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With respect to the multicenter trial mentioned by Apfalter et al, we would like to emphasize that out of 15 clinical samples tested in their study only 6 were obtained from carotid artery
compared with 46 samples tested in our study. Furthermore, no information on patients’ characteristics is provided. In the light of the recently shown high correlation between smoking and the presence of \textit{C pneumoniae} in atherosclerotic plaques (95% positive plaques in smokers versus 36% positive plaques in nonsmokers), this could well—at least in part—explain differences in prevalence of the pathogen. We have also discussed this fact in our article as well as the socioeconomic status of the patients as a confounding factor. Without this vital information it is not possible to assume that patients even at the same hospital must show similar prevalence values.

We are well aware of the published variation in the prevalence of \textit{C pneumoniae} DNA in atherosclerotic plaques. In fact, we also discuss this point in our article. However, we did not see the necessity to cite studies dealing with the reliability of PCR, because firstly this is not the major point of our article, and secondly we—at least in our opinion—had taken all necessary precautions and had performed proper controls in our study in order to prevent or to rule out cross contamination.

In conclusion, we feel that we have adequately addressed the points raised by Apfalter et al in their letter and we are confident that we have demonstrated that our data are valid. Finally, we wish to emphasize that we would have appreciated a discussion of our results based on scientific evidence but not based on the assumption that we lack experience in PCR and perform this technique without proper controls.

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