JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2002, p. 4428–4434 Vol. 40, No. 12
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.12.4428–4434.2002

Reliability of Nested PCR for Detection of *Chlamydia pneumoniae* DNA in Atheromas: Results from a Multicenter Study Applying Standardized Protocols

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Received 7 March 2002/Returned for modification 13 July 2002/Accepted 12 August 2002

The present multicenter study was designed to find explanations for the discrepancies in the reported rates of detection of *Chlamydia pneumoniae* DNA in endarterectomy specimens. Coded identical sets of (i) a *C. pneumoniae* DNA dilution series (panel 1; $n = 10$), (ii) spiked control tissue specimens (panel 2; $n = 10$ specimens, including 5 negative controls), and (iii) endarterectomy specimens (panel 3; 15 atheromas, 5 negative controls) were analyzed at four laboratories by three standardized DNA extraction methods in each laboratory and a nested touchdown PCR protocol targeting the *ompA* gene of *C. pneumoniae*. Panel 1 samples were correctly identified as positive to levels of 0.3 inclusion-forming units (IFU)/PCR mixture (100%) and 0.03 IFU/PCR mixture (50%). All negative controls were correctly reported as negative. Panel 2 samples were identified as *C. pneumoniae* positive to levels of 0.01 IFU/PCR mixture (100%) and 0.005 IFU/PCR mixture (91%), independent of the DNA extraction method used, and only one false-positive result was reported. For panel 3 samples, 5 of 240 (2%) analyses (in which DNA extractions and PCR were performed at the same laboratory) were positive; the positive specimens were from three endarterectomy specimens and two negative controls. After exchange of DNA extracts between laboratories, 13 of 15 atheroma samples were *C. pneumoniae* DNA positive in at least 1 of a series of 48 analyses per atheroma sample; however, the overall positivity rate did not exceed 5% (33 of 720 analyses) and therefore was lower than that for the negative controls (8%; 19 of 240 analyses). Not a single positive result could be achieved when all panel 3 extracts ($n = 240$ analyses) were reamplified by a 16S rRNA PCR followed by hybridization with a *C. pneumoniae*-specific probe. Statistical analyses demonstrated that positive results did not occur in an independent and random fashion and could most likely be explained by amplicon carryover at the nested PCR level as well as amplicon introduction during DNA extraction, but not by the patterns of distribution of very low target levels or a certain DNA extraction protocol. The results of studies by nested PCR for detection of the prevalence of *C. pneumoniae* will always be questionable.

During the past decade the association between *Chlamydia pneumoniae*, an important respiratory pathogen in humans (10, 11), and atherosclerosis (8) has continually been strengthened by direct detection of the organism in atherosclerotic tissues (21). Although there are no standardized PCR protocols, nested PCR has so far been the method most often used for this purpose. However, comparison of the data has been difficult because detection rates have varied considerably by protocol and individual testing center (4, 9, 12, 14–19, 21, 22, 24). Until now no systematic evaluation of various DNA extraction protocols and kits or of PCRs has been conducted for atherosclerotic tissue specimens. It is still not clear if methodological difficulties, a target distribution problem, various *C. pneumoniae* prevalences in different studies, or all of these factors account for the vast divergence of positive findings.

To date only two published studies have compared the results of detection methods conducted at different centers (1, 19). The rates of concordance of positive results in the study organized by Ramirez et al. (19) ranged between 9 and 82%. Since different parts of the atherosclerotic vessels were analyzed in the participating laboratories, a random distribution of *C. pneumoniae* within the plaques may explain some of the discrepancies. Recently, a multicenter trial was conducted to compare various DNA extraction methods and PCR protocols by using identical specimens (1), but no correlation between the detection rates and the sensitivities of the methods was found. While analyses performed with control tissues spiked with *C. pneumoniae* constantly showed similar results within the different laboratories, the reported rates of positivity for the atheroma panel varied between 0 and 60%, with the maximum rate of concordance for positive results being only 25% (1).

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As a continuation of that study, the present multicenter study was designed to find explanations for these rather unexpected results by keeping experimental variables to a minimum. This study specifically addresses the following questions. Does the DNA extraction protocol influence the overall rate of positivity for *C. pneumoniae* in atherosclerotic vessels? Given that the level of the target in a clinical sample is very low, does replicate PCR testing enhance sensitivity and therefore raise the overall positivity rate? Does contamination play a role, and if so, at what step does it occur?

MATERIALS AND METHODS

Study design. Four laboratories that participated in a previous study (1) received three experimental panels that were numbered by a participant-specific code and that consisted of aliquots of (i) a ready-to-use *C. pneumoniae* DNA dilution series (panel 1; for determination of the sensitivity of the nested touchdown PCR protocol of Tong and Sillis [23], which targets the *ompA* gene of *C. pneumoniae*), (ii) mock-infected control tissue specimens (panel 2; for comparison of the three DNA extraction methods performed independently at each laboratory), and (iii) endarterectomy samples (for extraction of the DNA from each sample at each center by the same three protocols used for panel 2 samples, followed by sharing of DNA among the laboratories and testing for *ompA* by nested PCR). In addition, the amount of DNA in all 240 panel 3 DNA extracts exchanged among the laboratories (20 samples were prepared at each of the four laboratories, with three DNA extraction methods used per sample) was measured (DyNA-Quant 200 fluorometer; Pharmacia, San Francisco, Calif.) and retested by using primers targeting the 16S rRNA gene of *C. pneumoniae*, followed by dot blot analysis (16) at laboratory A.

***C. pneumoniae* dilution series.** Serial dilutions of a frozen chlamydial stock preparation (strain MUL-1; M. Maass, Lübeck, Germany) were cultured in triplicate onto HEp-2 cells (CCL-23; American Type Culture Collection), and infectivity titers were determined 72 h after infection as described previously (20). To obtain purified *C. pneumoniae* elementary bodies for DNA preparation, stock chlamydiae were purified by differential centrifugation. To determine the corresponding numbers of chlamydial particles by immunofluorescence microscopy, aliquots of chlamydial dilutions were centrifuged onto lysin-coated coverslips. All experiments were done in triplicate. DNA from elementary bodies was extracted as described previously (2) and measured with a DyNA-Quant 200 fluorometer (Pharmacia).

Experimental panel 1. Each participant received 10 aliquots (50 μl each) of a 10-fold dilution of chlamydial DNA ($n = 6$) and water-containing negative controls ($n = 4$) ready for testing by PCR. The aliquots with DNA contained *C. pneumoniae* DNA at concentrations ranging from 1.3 μg/ml to 13 pg/ml (corresponding to 10^5 to 1 chlamydial particles/ml or 3×10^3 to 3×10^{-2} inclusion-forming units [IFU]/ml).

Experimental panel 2. Each participant received in triplicate 10 aliquots of *C. pneumoniae*-spiked porcine aortic tissue samples ($n = 5$; containing 10^2, 10^1, 1, 0.1, and 0.05 IFU [corresponding to 3.5×10^4 to 17.5 particles]/ml of aortic porcine homogenate, respectively) and unspiked ($n = 5$) porcine aortic tissue samples. The porcine aortic tissue samples had been homogenized to 20 mg/ml in Dulbecco's phosphate-buffered saline (Gibco, Life Technologies, Paisley, Scotland) and were prepared as described recently (1). Aliquots of 2 ml each were centrifuged ($15{,}000 \times g$ for 30 min) at 4°C, and pellets ready for DNA extraction were stored at −80°C.

Experimental panel 3. Frozen (−80°C) endarterectomy specimens ($n = 15$) obtained from patients with severe atherosclerosis and therefore undergoing vascular surgery (carotid artery, $n = 8$; femoral artery, $n = 3$; aneurysm of the abdominal aorta, $n = 4$) were thawed in one session, decalcified, and homogenized to 20 mg/ml in Dulbecco's phosphate-buffered saline with sterile tissue grinders, vortexed, immediately divided into 2-ml aliquots, centrifuged at 4°C ($15{,}000 \times g$ for 30 min), and refrozen as pellets at −80°C. Negative controls (porcine aortic tissue; $n = 5$) were prepared together with those used for panel 2. Thus, each participating laboratory received a total of 60 samples (specimens, $n = 45$; negative controls, $n = 15$; three aliquots of each specimen and control for three different DNA extractions).

No mock-infected tissue controls were included in panel 3, and neither the laboratory in which the specimens in the panel were prepared nor the pipettes used to prepare the specimens in the panel had ever been used for *C. pneumoniae*-related work. Panels 1 and 2 were prepared after the processing of negative controls and clinical specimens in a laminar-flow cabinet in a physically separated room. Experimental panels were stored at −80°C until they were shipped within 48 h on dry ice to the collaborating laboratories.

DNA extraction. DNA of identical tissue aliquots (40 mg of tissue for each of panels 2 and 3) were extracted by use of two commercial kits, the High Pure PCR template preparation kit (BM kit; suitable for extraction of DNA from 25 to 50 mg of tissue; Boehringer, Mannheim, Germany) and the QIAmp DNA Mini kit (Q kit; tissue protocol adjusted to 40 mg; Qiagen, Chatsworth, Calif.), according to the instructions of the manufacturers. DNA was also extracted by a conventional proteinase K phenol-chloroform protocol (K protocol) (2). The DNA was eluted or dissolved in a volume of 200 μl and divided into four 50-μl aliquots. One aliquot was tested by PCR at the same laboratory where the DNA extraction was performed, and the other three aliquots were sent to the other laboratories for analysis by PCR.

PCR. Each laboratory used identical amounts of template for PCR analysis: 10 μl of template (2 mg of tissue)/50 μl of first-round PCR mixture and 1 μl of amplicon/50 μl of the PCR mixture for the nested PCR step. Otherwise, the PCRs as well as the cycling conditions were as described previously (23). To check for the presence of PCR inhibitors that may have been present in the atheroma panel, all panel 3 DNA extracts prepared in each laboratory (60 at each laboratory) were run in duplicate; the samples tested were unspiked or spiked with *C. pneumoniae* DNA in an amount corresponding to that from about one to five elementary bodies. In case of inhibition the study protocol recommended retesting of a 1:10 dilution of the original DNA extract. The nested PCR products were separated by electrophoresis in 3% agarose gels at 80 V/h by using Tris-borate-EDTA buffer (pH 8.3) and were visualized with ethidium bromide. Negative results were considered or reported as true negative only if the DNA in the corresponding spiked sample could be amplified, all extraction-negative as well as in-house negative controls tested negative, and the positive controls (which contained the same small amount of *C. pneumoniae* DNA used to spike the samples) tested positive. The presence of amplicons 207 bp in length was considered a positive result for *C. pneumoniae* if all controls reacted as expected.

To minimize the risk of contamination, DNA extraction, PCR amplification, and electrophoresis were performed in separate rooms in all four laboratories. Each room had its own UV hood and set of pipettes, gloves, and disposable racks; and aerosol-resistant pipette tips were used. All reagents used were subdivided into small aliquots for each PCR run. Furthermore, the surface of every tube containing foreign panel 3 DNA extracts was decontaminated with sodium hypochlorite prior to handling in the area where PCR was carried out in each laboratory. To further avoid contamination, samples were always handled in the following order: clinical specimens (including the five blindly matched negative controls) as well as extraction-negative controls at every second position, in-house water containing negative controls at each fifth position for the first and nested PCRs, spiked specimens, in-house negative control, master mix control, and positive control. The same procedure had to be followed throughout the nested PCR setup.

Statistical analysis. The purpose of the statistical analysis was to analyze the distribution of positive results for negative controls and atheromas across different combinations of laboratories, PCR extractions, and amplification (panel 3). For every such combination a series of 5 negative controls and 15 atheroma samples was available. Therefore, the probability of observing k positive results among n samples tested ($n = 5$ and 15, respectively) is given by a binomial distribution, provided the probability for a positive result is constant and independent for each sample (i.e., provided that the probability of contamination is constant over all laboratories, DNA extractions, and the amplification assay for the negative samples and that the joint probability of detection of chlamydial DNA and contamination is constant for atheroma samples). Comparisons of the observed and expected distributions of positive results were done by Pearson's chi-square goodness-of-fit test.

RESULTS

Panel 1. The results for a blindly tested DNA dilution series are shown in Table 1. All four laboratories (100%) identified the *C. pneumoniae* DNA-containing controls as positive to a level of 3×10^1 IFU/ml (3×10^{-1} IFU/PCR mixture), and two laboratories (50%) detected 3 IFU/ml (3×10^{-2} IFU/PCR mixture). All analyses ($n = 16$) of the water-containing negative controls ($n = 4$) had a negative result in all laboratories (100%).

TABLE 1. Detection of *C. pneumoniae* DNA in a ready-to-use dilution series in four laboratories

Sample	Amt of chlamydiae/PCR mixture[a]			PCR result[b] by laboratory			
	No. of IFU	No. of EBs[c]	Amt of DNA	A	B	C	D
S1	30	1,000	1.3 ng	+	+	+	+
S2	0	0	0	−	−	−	−
S3	3	100	130 pg	+	+	+	+
S4	0	0	0	−	−	−	−
S5	0.3	10	13 pg	+	+	+	+
S6	0	0	0	−	−	−	−
S7	0.03	1	1.3 pg	+	−	−	+
S8	0	0	0	−	−	−	−
S9	0.003	0.1	130 fg	−	−	−	−
S10	0.0003	0.01	13 fg	−	−	−	−

[a] Amount of chlamydiae (IFU corresponding to elementary bodies or DNA) in a 10-μl amplified DNA extract/50 μl of first-round PCR mixture.
[b] +, reported as *C. pneumoniae* positive by nested PCR; −, reported as *C. pneumoniae* negative by nested PCR.
[c] EBs, elementary bodies.

Panel 2. *C. pneumoniae*-spiked tissue samples ($n = 5$; three aliquots of each sample) and unspiked tissue samples ($n = 5$; three aliquots of each sample) were analyzed, and the results are shown in Table 2. By use of the BM kit all four laboratories (100%) identified the spiked samples as positive to 0.5 IFU/ml (0.005 IFU/PCR mixture). Nineteen of 20 analyses of the negative controls ($n = 5$) were reported as negative (95%). By use of the Q kit, three laboratories (75%) identified the samples spiked with *C. pneumoniae* as positive to 0.5 IFU/ml (0.005 IFU/PCR mixture), and 1 laboratory identified the spiked samples as positive to 1 IFU/ml (0.01 IFU/PCR mixture). All 20 analyses of the negative controls ($n = 5$) had negative results (100%). By the noncommercially available K protocol, one laboratory failed to detect *C. pneumoniae* in any of the spiked control tissues, and the other three laboratories identified *C. pneumoniae* in the samples spiked with *C. pneumoniae* as positive to 0.5 IFU/ml (0.005 IFU/PCR mixture). The results for all negative controls (100%) were reported as negative.

TABLE 2. Detection of *C. pneumoniae* in aliquots of porcine aortic tissue samples in four laboratories by three different DNA extraction protocols

Sample	Amt of chlamydiae/PCR mixture[a]		PCR result by the following DNA extraction protocol for the indicated laboratory[c]											
	No. of IFU	No. of EBs[b]	BM kit				Q kit				K protocol			
			A	B	C	D	A	B	C	D	A	B	C	D
S1	10	3,500	+	+	+	+	+	+	+	+	+	+	+	−
S2	0	0	−	−	−	−	−	−	−	−	−	−	−	−
S3	1	350	+	+	+	+	+	+	+	+	+	+	+	−
S4	0	0	−	−	−	−	−	−	−	−	−	−	−	−
S5	0.1	35	+	+	+	+	+	+	+	+	+	+	+	−
S6	0	0	−	−	−	+	−	−	−	−	−	−	−	−
S7	0.01	3.5	+	+	+	+	+	+	+	+	+	+	+	−
S8	0	0	−	−	−	−	−	−	−	−	−	−	−	−
S9	0.005	1.75	+	+	+	+	+	−	+	+	+	+	+	−
S10	0	0	−	−	−	−	−	−	−	−	−	−	−	−

[a] Amount of chlamydiae (numbers of IFU corresponding to numbers of elementary bodies) in a 10-μl amplified DNA extract/50 μl of first-round PCR mixture.
[b] EBs, elementary bodies.
[c] +, reported as *C. pneumoniae* positive by nested PCR; −, reported as *C. pneumoniae* negative by nested PCR.

TABLE 3. Clinical evaluation of three different DNA extraction protocols for detection of *C. pneumoniae* DNA in endarterectomy specimens[a]

Sample[b]	No. of *C. pneumoniae* positive PCR results by the following DNA extraction protocol for the indicated laboratory:											
	BM kit				Q kit				K protocol			
	A	B	C	D	A	B	C	D	A	B	C	D
Atheroma ($n = 15$)	0	0	2[c]	0	0	0	0	1[d]	0	0	2[c]	0
Negative control ($n = 5$)	0	0	2	0	0	0	0	0	0	0	0	0

[a] The PCR results reported from the four laboratories are for DNA extracts exclusively prepared in each laboratory.
[b] An endarterectomy specimen or porcine aortic tissue (negative controls) was reported as *C. pneumoniae* positive when extracts prepared in each laboratory were tested by PCR.
[c] Concordant results for two endarterectomy specimens (arteria carotis and arteria femoralis).
[d] Endarterectomy specimen (arteria femoral) different from the arteria femoralis described in note *c*.

Panel 3. Clinical evaluation. Table 3 displays only the PCR results obtained with the DNA extracts prepared in each laboratory. Of a total of 240 analyses (180 analyses with 15 endarterectomy specimens and 60 analyses with 5 negative controls), 7 (3%) were reported to be positive for *C. pneumoniae*: 5 positive analyses (2%) stemmed from 3 endarterectomy specimens (for laboratory C, concordant results for one arteria carotis specimen and one arteria femoralis specimen with the BM kit and by the K protocol; for laboratory D, one positive arteria femoralis specimen with the Q kit;) and 2 (0.8%) stemmed from negative controls (for laboratory C, BM kit). Laboratories A and B did not report any positive results from their own atheroma extracts and identified all negative controls as negative.

Subsequently, DNA extracts (three extracts per sample per laboratory) were exchanged among the four laboratories, and the PCR results are summarized in Table 4. Taken together, each sample was analyzed 48 times (12 DNA extracts per sample tested by PCR at four laboratories each; in summary, 960 analyses [720 and 240 analyses from supposedly identical aliquots of 15 endarterectomy samples and 5 negative controls, respectively]). In summary, 33 of 720 analyses (5%) of endarterectomy specimens were positive for *C. pneumoniae*, but none of them was positive by more than 5 of 48 analyses, showing a concordant result for positivity of only 10%. At least 1 of 48 analyses reported a positive result for 13 endarterectomy specimens (86%). Only two atheromas were reported to be negative by all 48 analyses. Compared to the results for the endarterectomy specimens, the ratio of positivity was greater for the negative controls (19 of 240; 8%) with at least 2 of 48 analyses reporting positive results for all five negative controls.

Considering the different DNA extraction methods, the distributions of positive results for *C. pneumoniae* were as follows: for the BM kit, 22 of 240 (9%) endarterectomy samples and 11 of 80 (14%) negative controls; for the Q kit, 5 of 240 (2%) endarterectomy samples and 5 of 80 (6%) negative controls; for the K protocol, 6 of 240 (3%) endarterectomy samples and

TABLE 5. Overall *C. pneumoniae* positivity rates for endarterectomy samples (panel 3) and negative controls reported by each laboratory (laboratories A to D) by the origin of the DNA extracts tested and the DNA extraction protocol used

Laboratory	Total no. of positive[a] analyses	No. of positive[b] atheromas	No. of false-positives analyses[c]	No. of positive analyses (origin[d] of extracts)		DNA extraction method or protocol used
				Atheroma	Negative controls[c]	
A	4	3	1	1 (C)		BM
				2 (C)	1 (C)	K
B	16	13	3	11 (C)	2 (C)	BM
				2 (C)	1 (C)	K
C	17	11	6	1 (A)	1 (A)	BM
				1 (B)	1 (B)	BM
				2 (C)	2 (C)	BM
				1 (D)	1 (D)	BM
				2 (A)	1 (A)	Q
				2 (B)		Q
				2 (C)		K
D	15	6	9	1 (A)	1 (A)	BM
				4 (C)	3 (C)	BM
					3 (A)	Q
					1 (C)	Q
				1 (D)		Q
					1 (C)	K

[a] Number of samples reported as *C. pneumoniae* DNA positive of a total of 960 analyses.
[b] Number of endatherectomy specimens reported as *C. pneumoniae* DNA positive of a total of 720 analyses.
[c] Porcine aortic tissue samples (negative controls) reported as *C. pneumoniae* DNA positive of a total of 240 analyses.
[d] DNA was extracted at laboratory A, B, C, or D.

3 of 80 (4%) negative controls. Table 5 shows the overall positivity ratio achieved by each laboratory in the context of the origins of the DNA extracts.

All 240 exchanged panel 3 DNA extracts retested at laboratory A by a 16S rRNA nested PCR (for laboratory H, the detection limit for spiked vascular tissue samples was 1 elementary body/PCR mixture [1]) followed by dot blot hybridization were negative for *C. pneumoniae* DNA. The mean DNA contents of these extracts measured at laboratory A were 0.12, 0.11, 0.04, and 0.07 μg/μl for laboratories A, B, C, and D, respectively. With respect to the various DNA extraction methods, the mean DNA content in extracts obtained with the BM kit was 0.11 μg/μl (range of mean values, 0.035 to 0.19 μg/μl at laboratories C and A, respectively), the mean DNA content in extracts obtained with the Q kit was 0.11 μg/μl (range of mean values, 0.063 to 0.15 μg/μl at laboratories C and A [and laboratory B], respectively), and the mean DNA content in extracts obtained by the K protocol was 0.03 μg/μl (range of mean values, 0.027 to 0.04 μg/μl at laboratories C and D, respectively).

Statistics. Analysis of the frequency distribution of positive results for negative controls, assuming a contamination probability of 8% (the results for negative controls reported as positive), and comparison with the binomial revealed a goodness-of-fit chi-square statistic of 3.08 (2 degrees of freedom [df]), which was almost significant ($P = 0.215$). Under the assumption of constancy of contamination probability and independence, among the five negative controls tested there

TABLE 4. Clinical evaluation of three different DNA extraction protocols for detection of *C. pneumoniae* DNA in endarterectomy specimens after exchange of DNA extracts among four laboratories (laboratories A to D)[a]

Panel 3 sample	No. of *C. pneumoniae*-positive PCR results by method and laboratory for:																											
	DNA extraction performed at laboratory A								DNA extraction performed at laboratory B								DNA extraction performed at laboratory C								DNA extraction performed at laboratory D							
	BM				Q				K				BM				Q				K				BM				Q				K	
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Atheroma ($n = 15$)	0	0	1	1	0	0	2	0	0	0	0	0	0	1	0	0	0	0	2	0	0	0	0	1	11	2	4	0	0	0	0	2	2	2	0	0	0	0	1	0	0	0
Negative control ($n = 5$)	0	0	1	1	0	0	1	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	2	2	3	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0

[a] Each sample was tested by PCR 48 times, with a total of 960 analyses performed: 720 and 240 analyses with identical aliquots from 15 endarterectomy samples and 5 negative controls, respectively.

were slightly too many series with no positive results and too few with just one positive result. For the endarterectomy samples, however, the deviation from the binomial distribution was highly significant (chi-square = 11.99; df = 3; $P = 0.007$). In particular, the probability (under the assumption of constancy and independence) that more than five positive atheroma specimens would be observed among 15 atheroma specimens is 3 to 100,000 given the overall rate of positivity (5%). Note that one of the series had 11 positive specimens. From these results the assumption of constancy and independence of positive specimens can be refuted. Thus, the observation of a positive result does not occur in an independent and random fashion. If this had been due to the presence of chlamydial DNA in some of the samples but not in others, the frequency distribution of positive results for the 15 specimens across the 48 tests performed should likewise have deviated from the binomial. However, this was not the case (chi-square = 2.589; df = 4; $P = 0.629$).

DISCUSSION

This study was originally undertaken because the demand for a standardized PCR assay for the detection of *C. pneumoniae* from clinical specimens has evolved in recent years (5, 7). Hence, four laboratories applied the nested PCR of Tong and Sillis (23) to DNA extracts prepared by different extraction methods under standardized conditions and also interchanged DNA extracts.

The PCR results of the present study achieved with purified *C. pneumoniae* DNA were uniform, highly sensitive (0.3 to 0.03 IFU/PCR mixture), specific, and reproducible at a very low target level at each laboratory and therefore allowed the direct comparison of the three DNA extraction protocols applied to mock-infected controls. With the exception of the in-house K protocol and one false-positive result at laboratory D, even lower detection limits (0.005 IFU/PCR mixture) were reported for the mock-infected controls. It could be that the presence of tissue DNA acted as a carrier and increased the total yield of the *C. pneumoniae* targets.

However, the results for the endarterectomy samples are not altogether unexpected considering the results of the previous study (1), but they are nevertheless disappointing.

If the focus is exclusively on data derived from analyses done with extracts prepared by each laboratory (Table 3), only 7 (3%) of 240 analyses reported positive results for *C. pneumoniae*: laboratory C reported four positive results (2%) for two endarterectomy specimens (specimens 3 and 14) but two false-positive results for negative controls as well, and laboratory D found specimen 2 to be positive with the Q kit. Laboratories A and B did not report any positive results from the atheroma extracts that they prepared in their own laboratories and identified all negative controls as negative. In light of the false-positive results obtained with negative controls at laboratory C, none of these positive results for endarterectomy specimens should be considered valid.

After exchange of extracts, at least 1 of 48 analyses was positive for 13 endarterectomy specimens (86%), a finding which could be in accordance with the fact that the target level was extremely low, but the distribution of positive findings did not follow a random distribution (even if the calculation was done with a background false-positivity rate of 8%). Therefore, an interpretation that nearly 90% of the specimens were positive (e.g., at a very low level) is not very convincing and is statistically very unlikely, although much higher PCR positivity rates have been published for specimens with low levels of target DNA, such as atherosclerotic tissues and peripheral blood mononuclear cells with only triplicate or even no repetitive testing at all (3, 4, 6, 12–14, 17, 25). Again, in this study the deviation of the number of positive results from the binomial distribution within each combination of laboratory, PCR extraction, and PCR amplification could have been because some specimens were truly positive. However, in this case the distribution calculated over specimens should also deviate from a binomial distribution. This was definitely not the case. Therefore, positivity is much more likely due to other factors than the presence of *C. pneumoniae* DNA in atheromas in the present study. Contamination, especially amplicon carryover in the nested PCR, is known to be an insidious problem, and it is possible that the literature in the field is biased by false-positive PCR results. This and the fact that studies with negative findings are less likely to be published could bias conclusions drawn from systematic reviews and meta-analyses.

In the present study, only two atheroma samples (from 48 analyses) were reported to be completely negative, which was not true for the negative controls, with a minimum of two analyses per 48 testing attempts reporting positive results for all five negative controls. Importantly, the positivity rate was even higher for negative controls than for endarterectomy samples. It is obvious that there was a contamination problem, but where and at what step did it occur?

The only laboratory that reported false-positive results at a time when no foreign extracts had been tested was laboratory C, and most—but not all—positive findings stemmed from extracts provided from that laboratory (34 of 52 analyses [65%]; Table 5); e.g., all 16 analyses with positive results reported by laboratory B stemmed from extracts prepared at laboratory C. On the other hand, laboratory C itself reported a lower positivity rate, and laboratory A reported only a single positive specimen among the same extracts. The occurrence of amplicon carryover at laboratory B during nested PCR could also explain the 13 positive samples tested in a series (Table 4). Anyhow, occurrences of contamination seem to be randomly dispersed among the results at a frequency below 2%, and cases of clustered contamination were detected in which almost all samples tested were positive. This points to a heterogeneity of contamination, with the most critical steps throughout the whole procedure being the nested step of the PCR itself as well as *ompA* amplicon introduction during DNA extraction. The fact that not a single positive sample could be detected when the extracts were retested by a PCR assay targeting a different gene (with the same detection limit for *C. pneumoniae* DNA for this kind of specimen) further supports the hypothesis of *ompA* amplicon contamination, as does the overall distribution of positive findings for laboratory C itself and laboratory D (Table 5).

Ong et al. (18) associated the high numbers of positive findings in the previous study (1) with the application of the BM kit (whose use requires a large number of manipulation steps, resulting in a high rate of false-positive results). However, we are of the opinion that the choice of DNA extraction

kit is of minor importance. The Q kit, e.g., requires as many manipulation steps as the BM kit (six spin steps and opening of vials on 11 occasions until elution of DNA), but only a few or even no positive results were achieved with that kit in the present study and the previous study (1) as well as in the study conducted by Ong et al. (18). In addition, in the present study both kits recovered similar amounts of DNA, and although laboratory C especially appeared to be more efficient in recovering DNA with the BM kit, these extracts revealed the smallest amounts of DNA compared to the amounts detected by the other laboratories when they used the BM kit.

In this study experienced laboratories obviously had problems with contamination. It stands to reason that all four laboratories performed DNA extraction, first and nested PCR amplifications, and electrophoresis in separate rooms equipped with their own UV hoods, sets of pipettes, disposable racks, and aerosol-resistant pipette tips. Furthermore, all participants were requested to decontaminate tubes containing external foreign DNA before they opened the tubes and performed PCR at each laboratory and to include additional water-containing negative controls in the first PCR and at the nested PCR level as well as negative controls following the whole DNA extraction procedure. Since the *ompA* PCR assay applied in the present study consisted of a nested step, inactivation of the first PCR product (containing dUTP instead of dTTP) by the enzyme uracil *N*-glycosylase as well as isopsoralen treatment could not be performed, since both methods would have destroyed the template for the nested PCR step. Anyhow, all attempts were used to reduce contamination, and PCR results were reported only if all in-house negative controls as well as positive controls reacted as expected; however, contamination did happen and came to light only because of the study design.

These data demonstrate the limits of nested PCR, which might be necessary for detection of an organism assumed to be present only at a very low level but whose use is connected with a high risk of contamination. As long as methods like nested PCR are used, we recommend that several negative control tissue samples always be included for each specimen analyzed. Control samples should contain enough carrier DNA to detect low-level amplicon carryover and should be used instead of negative controls containing water only, and controls must be used throughout the procedure, from DNA extraction to detection of the PCR product. By this procedure contamination can at least be identified immediately. In addition, external quality control should be aimed at every laboratory performing nested PCR on clinical samples. Furthermore, repeated analyses (including repeated DNA extractions) can be performed to further increase specificity. However, the drawback of this procedure is that it is labor intensive and expensive. Therefore, alternative highly sensitive methods such as real-time PCR should be established on a commercial basis as soon as possible because they are less prone to contamination and, in addition, allow target quantification.

The main conclusion of the present study is that although the intention was to control the methodology as best as possible, the question of cross-contamination remains whenever a nested PCR is performed. In this sense, the results of studies that use nested PCR to determine the prevalence of *C. pneumoniae* will always be questionable.

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