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Research article

Evaluation of five DNA extraction methods for purification of DNA from atherosclerotic tissue and estimation of prevalence of *Chlamydia pneumoniae* in tissue from a Danish population undergoing vascular repair

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Abstract

Background: To date PCR detection of *Chlamydia pneumoniae* DNA in atherosclerotic lesions from Danish patients has been unsuccessful. To establish whether non-detection was caused by a suboptimal DNA extraction method, we tested five different DNA extraction methods for purification of DNA from atherosclerotic tissue.

Results: The five different DNA extraction methods were tested on homogenate of atherosclerotic tissue spiked with *C. pneumoniae* DNA or EB, on pure *C. pneumoniae* DNA samples and on whole *C. pneumoniae* EB. Recovery of DNA was measured with a *C. pneumoniae*-specific quantitative real-time PCR. A DNA extraction method based on DNA-binding to spin columns with a silica-gel membrane (DNeasy Tissue kit) showed the highest recovery rate for the tissue samples and pure DNA samples. However, an automated extraction method based on magnetic glass particles (MagNA Pure) performed best on intact EB and atherosclerotic tissue spiked with EB. The DNeasy Tissue kit and MagNA Pure methods and the highly sensitive real-time PCR were subsequently used on 78 atherosclerotic tissue samples from Danish patients undergoing vascular repair. None of the samples were positive for *C. pneumoniae* DNA. The atherosclerotic samples were tested for inhibition by spiking with two different, known amounts of *C. pneumoniae* DNA and no samples showed inhibition.

Conclusion: As a highly sensitive PCR method and an optimised DNA extraction method were used, non-detection in atherosclerotic tissue from the Danish population was probably not caused by use of inappropriate methods. However, more samples may need to be analysed per patient to be completely certain on this. Possible methodological and epidemiological reasons for non-detection of *C. pneumoniae* DNA in atherosclerotic tissue from the Danish population are

discussed. Further testing of DNA extraction methods is needed as this study has shown considerable intra- and inter-method variation in DNA recovery.

Background

Chlamydia pneumoniae is an important cause of human respiratory tract diseases [1]. The organism has also been associated with atherosclerosis and thromboembolic events by use of seroepidemiology and direct detection of the organism in atherosclerotic plaques [2]. Although the presence of *C. pneumoniae* DNA has been observed in atherosclerotic lesions, the pathological or clinical significance is unknown and difficult to elucidate. One of the keys to answering these questions could be identifying differences between populations in which the organism is detected and populations in which it is not. In contrast to populations outside Denmark [3–5] attempts to detect *C. pneumoniae* in atherosclerotic tissue obtained from Danes have not been successful [6,7].

The issue of methodology is highly relevant when considering whether or not Danes are infected by *C. pneumoniae* in vascular tissue. A recent study [8] showed major interlaboratory variation between *C. pneumoniae* PCR and DNA extraction methods even when they were applied on the same set of samples. A reasonable assumption could therefore be that former Danish studies with negative findings used PCR tests that were too insensitive to allow detection or that DNA extraction methods were insufficient to remove inhibitors.

In order to confirm and justify the previous Danish findings we conducted a study to find the DNA preparation method with the highest obtainable DNA recovery and inhibitor removal. We examined five different methods for purifying DNA from atherosclerotic tissue. The recovery of DNA from three different sample types was tested with a quantitative real-time PCR specific for *C. pneumoniae* [9,10]. The best DNA purification method was selected and used in combination with the *C. pneumoniae*-specific real-time quantitative PCR to assess the prevalence of *C. pneumoniae* DNA in atherosclerotic tissue samples from patients undergoing vascular repair. Inhibition in the samples was tested by spiking aliquots of the atherosclerotic tissue DNA samples with a known amount of *C. pneumoniae* DNA before subjecting them to PCR.

Results

By a literature search we selected the five most commonly used DNA extraction methods; 1) a standard phenol/chloroform purification method followed by an ethanol precipitation, 2) same method as 1) but with a cetyltrimethylammonium bromide (CTAB) precipitation step included (at high NaCl CTAB precipitates polysaccharides and proteins) [11], 3) the DNeasy Tissue kit from Qiagen, a silica-gel column-based method, 4) the Easy-DNA kit from Invitrogen [12] 5) and the MagNA Pure LC Instrument that provides automated DNA purification based on magnetic glass particles. DNA purification methods were tested on the following sample panels 1) samples with purified *C. pneumoniae* genomic DNA in concentrations of 10^5,10^3, 10 copies/μl and a negative control, 2) spiked aorta homogenate samples (10 mg tissue pr. sample) with *C. pneumoniae* genomic DNA concentrations of 10^5, 10^3, 10 copies/μl and a negative control. 3) Purified, intact *C. pneumoniae* EB in dilutions 1.2×10^{-4}, 1.2×10^{-5}, 1.2×10^{-6}, 1.2×10^{-8} and a negative control containing PBS only (corresponding to approximate EB concentrations of 2000, 200, 20, 0.2 and 0 EB/μl). 4) Aorta homogenate samples spiked with intact *C. pneumoniae* EB in dilutions 1.2×10^{-4}, 1.2×10^{-5}, 1.2×10^{-6}, 1.2×10^{-8} and a negative control (corresponding to approximate EB concentrations of 2000, 200, 20, 0.2 and 0 EB/μl). See the methods section for a detailed description of the samples. Purified *C. pneumoniae* genomic DNA was used for spiking of sample panel 1 because the exact amount of DNA input is known as opposed to the *C. pneumoniae* EB, which are difficult to quantify. Consequently, copies/μl refer to genome copy number. EB were also used alone and for spiking of aorta homogenate to test the methods for their capability of recovering DNA from intact bacteria. To simplify determination of how much DNA was recovered the input and output volume (100 μl) for the DNA extraction was the same for each sample. After DNA extraction the amount of recovered DNA was measured with a *C. pneumoniae*-specific real-time quantitative PCR on 2 μl of the output volume. Table 1 shows input copies/μl, mean output copies/μl for two replicates, and the recovery percentage for the pure *C. pneumoniae* genomic DNA samples and aorta samples spiked with *C. pneumoniae* genomic DNA. Table 2 shows input EB dilution/ concentration, mean output copies/μl for two replicates, and the relative recovery percentage for pure *C. pneumoniae* EB samples and aorta samples spiked with *C. pneumoniae* EB.

Pure DNA samples

All negative samples were correctly identified as negative, meaning that no contamination was introduced during purification. Four methods (phenol/chloroform, The DNeasy Tissue kit, Easy-DNA kit, MagNA Pure) were able to recover *C. pneumoniae* DNA from the lowest concentration sample (input concentration 10 copies/μl) with output concentrations ranging from 0.6–8 copies/μl (Table

Table 1: Five DNA extraction methods used on pure *C. pneumoniae* DNA and aorta tissue samples spiked with *C. pneumoniae* DNA For each sample type different DNA input concentrations were used, these are displayed in the first column. DNA input concentration was known as pure genomic *C. pneumoniae* DNA was used for preparation of samples. For each method and sample the mean output *C. pneumoniae* genomic DNA concentration and standard deviation (SD) is displayed for two replicates as determined with the quantitative *pmp4* LightCycler PCR. In addition percentage of recovered DNA is calculated (% Rec.). Standard deviations (SD) are also shown for the recovery percentages.

		phenol/chloroform				phenol/chloroform +CTAB				DNeasy Tissue kit				Easy-DNA kit				Magna Pure			
	Input copies /µl	Mean output copies/ µl	SD of Mean	% Rec.	SD of % Rec.	Mean output copies/ µl	SD of Mean	% Rec.	SD of % Rec.	Mean output copies/ µl	SD of Mean	% Rec.	SD of % Rec.	Mean output copies/ µl	SD of Mean	% Rec.	SD of % Rec.	Mean output copies/ µl	SD of Mean	% Rec.	SD of % Rec.
Pure *C. pneumoniae* DNA samples	0	0	0	-	-	0	0	-	-	0	0	-	-	0	0	-	-	0	0	-	-
	10	1.79	0.213	17.9	2.1	0	0	0.0	0	8.08	1.21	80.8	12.1	0.661	0.520	6.6	5.2	4.60	0.377	46.0	3.8
	10^3	296	15.4	29.6	1.5	11.8	3.20	1.2	0.3	658	77.7	65.8	7.8	48.9	14.5	4.9	1.4	508	49.0	50.8	4.9
	10^5	2.09×10^4	2.55×10^3	20.9	2.5	728	107	0.7	0.1	7.54×10^4	1.83×10^4	75.4	18.4	3.10×10^3	1.83×10^3	3.1	1.8	5.58×10^4	7.54×10^3	55.8	7.5
	av rec %	-	-	**22.8**	5.7	-	-	**0.6**	0.6	-	-	**74.0**	12.4	-	-	**4.9**	3.0	-	-	**50.9**	6.2
Aorta tissue samples spiked with *C. pneumoniae* DNA	0	0	0	-	-	0	0	-	-	0	0	-	-	0	0	-	-	0	0	-.	-
	10	0	0	0.0	0	1.31	0.240	13.1	2.4	4.56	4.19	45.6	42.0	6.34	1.50	63.4	15.0	1.83	0.622	18.3	6.2
	10^3	70.8	32.7	7.1	3.3	13.0	6.46	1.3	0.6	285	23.9	28.5	2.4	255	13.7	25.5	1.4	82.0	6.08	8.2	0.6
	10^5	5.00×10^3	2.55×10^3	5.0	2.4	1.40×10^3	611	1.4	1.0	2.37×10^4	502	23.7	0.5	3.30×10^4	7.53×10^3	33.0	7.5	1.61×10^4	1.06×10^4	16.1	10.6
	av rec %	-	-	**4.0**	3.7	-	-	**5.3**	6.2	-	-	**32.6**	21.4	-	-	**40.6**	19.4	-	-	**14.2**	7.3
	overall avrec %	-	-	**13.4**	10.8	-	-	**3.0**	4.8	-	-	**53.3**	27.3	-	-	**22.8**	22.9	-	-	**32.5**	20.2

1). For an input of 10^3 copies/µl output concentrations ranged from 12–658 copies/µl and for an input of 10^5 copies/µl output concentrations ranged from 728–7.5×10^4 copies/µl (Table 1). For all input DNA concentrations the DNeasy Tissue kit showed the highest output concentration. Consequently, for the pure *C. pneumoniae* DNA samples the DNeasy Tissue kit had the best average recovery percentage (74.0%) followed by the MagNA pure with an average recovery percentage of 50.9%. All five methods showed approximately 10% intra-method difference between the highest and lowest recovery percentages.

Homogenised aorta tissue spiked with DNA

All negative samples were correctly identified. Four methods (phenol/ chloroform + CTAB, The DNeasy Tissue kit, Easy-DNA kit, MagNA Pure) recovered *C. pneumoniae* DNA from the lowest input concentration sample (10 copies/µl) with output concentrations ranging from 1.3–6.3 copies/µl (Table 1) and the Easy-DNA kit had the highest output concentration. For an input of 10^3 copies/µl output concentrations ranged from 13–285 copies/µl and the DNeasy Tissue kit had the highest output concentration. For an input of 10^5 copies/µl output concentrations ranged from 1.4×10^3-3.3×10^4 copies/µl and the Easy-DNA kit had the highest output concentration. The range of recovery percentages obtained with one method was larger than for the pure DNA samples. For the spiked aorta samples the Easy-DNA kit had the best recovery percentage (40.6%) and the DNeasy Tissue kit the second best (32.6%).

Purified EB samples

All negative samples were correctly identified. No methods recovered *C. pneumoniae* DNA from the lowest EB dilution 1.2×10^{-8} (~0.2 EB/µl), but all methods recovered *C. pneumoniae* DNA from 1.2×10^{-6}, 1.2×10^{-5} and 1.2×10^{-4} dilutions (~2000, 200, 20 EB/µl) (Table 2). Recovery percentages were calculated relatively to the method performing best. The MagNA Pure method performed best and showed the highest output concentrations for all dilutions. The DNeasy Tissue kit showed relative recovery percentages from 79.8–95.3%. The Easy-DNA kit showed the lowest relative recovery percentages from 1.5–5.3%. The phenol/chloroform+CTAB method showed the second lowest recovery percentages (11.4–35.8%) and the phenol/chloroform the third lowest recovery percentages (20.2–42.8%) (Table 2).

Homogenised aorta tissue spiked with purified EB

All negative samples were correctly identified. Two methods recovered *C. pneumoniae* DNA from the lowest dilution 1.2×10^{-8} (~0.2 EB/µl); phenol/chloroform + CTAB

Table 2: Five DNA extraction methods used: pure intact *C. pneumoniae* EB and aorta tissue samples spiked with *C. pneumoniae* EB For each sample type different EB input dilutions were used, these are displayed in the first column. Shown are also a rough estimate of the EB concentrations as determined by real-time PCR. For each method and sample the mean output *C. pneumoniae* genomic DNA concentration and standard deviation (SD) is displayed for two replicates as determined with the quantitative *pmp4* LightCycler PCR. In addition relative recovery percent is calculated relative to the best method, which in this case was the MagNA Pure method (% Rel. rec.). Additionally standard deviations (SD) are also shown for the relative recovery percentages.

		phenol/chloroform					phenol/chloroform + CTAB				DNeasy Tissue kit				Easy-DNA kit				Magna Pure			
	EB Dilution	Appr. EB conc. EB/µl	Mean output copies/ µl	SD of Mean	% Rel. rec.	SD of % Relative rec.	Mean output copies/ µl	SD of Mean	% Rel. rec.	SD of % Relative rec.	Mean output copies/ µl	SD of Mean	% Rel. rec.	SD of % Relative rec.	Mean output copies/ µl	SD of Mean	% Rel. rec.	SD of % Relative V	Mean output copies/ µl	SD of Mean	% Rel. rec.	SD of % Relative rec.
C. pneumoniae EB samples	negative	0	0	0	-	-	0	0	-	-	0	0	-	-	0	0	-	-	0	0	-	-
	1.2×10^{-8}	0.2	0	0	-	-	0	0	-	-	0	0	-	-	0	0	-	-	0	0	-	-
	1.2×10^{-6}	20	3.76	4.60	42.8	52.4	1.38	0.410	15.8	4.7	8.36	3.59	95.3	40.9	0.464	0.160	5.3	1.8	8.78	0.005	100	0.06
	1.2×10^{-5}	200	28.6	29.9	27.0	28.2	38.0	44.7	35.8	42.2	96.2	19.3	90.8	18.2	1.81	1.24	1.7	1.2	106	38.3	100	36.2
	1.2×10^{-4}	2000	248	208	20.2	17.0	140	60.2	11.4	5.0	982	250	79.8	20.3	18.5	21.7	1.5	1.8	1.23×10^3	331	100	26.9
	av rec %	-	-	-	**30.0**	29.5	-	-	**21.0**	22.3	-	-	**88.6**	23.1	-	-	**2.8**	2.3	-	-	**100**	20.2
EB spiked aorta tissue samples	negative	0	0	0	-	-	0	0	-	-	0	0	-	-	0	0	-	-	0	0	-	-
	1.2×10^{-8}	0.2	0	0	0	0	0.219	0.310	18.0	25.4	0	0	0	0	0	0	0	0	1.22	1.72	100	141.3
	1.2×10^{-6}	20	10.1	3.92	53.2	20.7	14.3	7.05	75.6	37.3	13.9	4.82	73.7	25.5	2.33	0.184	12.3	1.0	18.9	2.86	100	15.1
	1.2×10^{-5}	200	50.3	24.4	44.5	21.6	125	7.14	110.5	6.3	66.3	30.2	58.7	26.8	29.5	1.11	26.1	1.0	113	0.530	100	0.5
	1.2×10^{-4}	2000	632	177	49.0	13.7	648	585	50.3	45.3	869	208	67.3	16.2	191	128	14.8	9.9	1.29×10^3	247	100	19.2
	av rec %	-	-	-	**36.7**	26.0	-	-	**63.6**	43.7	-	-	**49.9**	34.8	-	-	**13.3**	10.6	-	-	**100**	54.2
	overall av rec %	-	-	-	**33.8**	26.7	-	-	**45.3**	41.2			**66.5**	35.4			**8.8**	9.6			**100**	41.7

and the MagNA Pure method (Table 2). From all other dilutions (~2000, 200, 20 EB/µl) (except one; 1.2×10^{-5}, where the phenol/chloro-form+CTAB method showed higher recovery) the MagNA Pure method also showed highest output DNA concentrations and thereby the best performance. The phenol/chloroform+CTAB method showed from 18.0–110.5% relative recovery and the DNeasy Tissue kit showed between 58.7–73.7% relative recovery. The phenol/chloroform (44.5–53.2%) and Easy-DNA kit methods (12.3–26.1%) showed the lowest relative recovery percentages (Table 2). For the two best methods (MagNA Pure and DNeasy) the whole process from DNA extraction to PCR detection had a sensitivity of less than approximately 20 EB/µl (concentration in samples before DNA extraction) (Table 2).

Combined test results

When comparing recovery percentages for the pure DNA samples and the aorta samples spiked with DNA, it is seen that there was greater intra-method variation for the spiked aorta samples. For two methods (phenol/chloroform+CTAB and Easy-DNA kit) the recovery percentage was generally lower for the pure DNA samples (Table 1). When the overall recovery percentage was calculated for all samples for each method, the DNeasy Tissue kit had the best recovery percentage (53.3%) and MagNA Pure the second best (32.5%) (Table 1). On the pure EB samples and aorta samples spiked with EB, the MagNA Pure method performed best and the DNeasy Tissue kit second best (Table 2). For two methods the phenol/ chloroform+CTAB and Easy-DNA kit performance was best on aorta samples spiked with EB analogous to the situation on aorta samples spiked with pure DNA. On the basis of these combined results the DNeasy Tissue kit and the MagNA Pure methods were chosen for tests on patient samples.

Patient samples

The DNeasy Tissue kit and the MagNA Pure method were tested for purification of DNA from 78 atherosclerotic samples. None of the 2 × 78 DNA preparations were positive for *C. pneumoniae* and all samples were positive for human DNA. The average human DNA concentration in the samples purified with the DNeasy Tissue kit was 1.1×10^4 copies/µl (SD = 1.6×10^4, range 6.2×10^2 to 1.1×10^5 copies/µl). The average human DNA concentration in samples purified with the MagNA Pure was 1.0×10^4 copies/µl (SD = 1.1×10^4 copies/µl, range 18 to 5.4×10^4 copies/µl). As an inhibition check all samples purified with both methods were spiked with two different, known concentrations of *C. pneumoniae* genomic DNA (10^3 copies/µl and 10 copies/µl). Subsequently, the spiked samples were subjected to real-time quantitative PCR. Here we found that both concentrations could be detected in all 78 samples using both extraction methods. The average *C. pneumoniae* concentration in the 78 samples purified with the DNeasy tissue kit and spiked with 10 copies/µl were 14.2 copies/µl (SD = 5.4) and in samples spiked with 10^3 cop-

ies /µl it was 1.1×10^3 copies/µl (SD = 185). The average *C. pneumoniae* concentration in the 78 samples purified with the MagNA Pure method and spiked with 10 copies/µl were 17.9 copies/µl (SD = 9.5) and in samples spiked with 10^3 copies /µl it was 1.4×10^3 copies/µl (SD = 287).

Discussion

DNA purification methods

Two methods (phenol/chloroform+CTAB and Easy-DNA kit) performed better on the spiked homogenates than on pure *C. pneumoniae* DNA or EB (Table 1 and 2). It could reflect that in general these two purification methods perform better on the tissue samples as the purification kinetics is probably different in the two sample types. The Easy-DNA kit was used strictly as recommended by the manufacturers and not with the improvements done by Meijer et al. [12]. They replaced the ethanol precipitation by further purification with silica particles and it was scaled up to enable DNA purification from 300 mg of tissue. This gave better sensitivity [12], so if we used their modification, the Easy-DNA kit might have performed better. Loss of DNA could be caused by precipitation, phenol/chloroform or chloroform extraction steps, as these steps are prone to DNA loss. This is also indicated by the fact that Meijer et al. [12] could improve the Easy-DNA method by replacing the precipitation step with silica-particles. The phenol/chloroform+CTAB method contains an additional precipitation step (CTAB) compared to conventional phenol/chloroform extraction, which may account for its relatively poor performance with pure DNA. The three remaining methods (phenol/chloroform, DNeasy Tissue kit and MagNA Pure) all showed reduced recovery for the homogenates. The DNeasy Tissue kit showed the best recovery on both pure DNA samples and aorta homogenates spiked with DNA. On purified EB and aorta homogenate spiked with EB the MagNA Pure method performed best. That two different methods were found to be best, probably reflects the different nature of the samples and underlines the importance of using whole bacteria and not just pure DNA for testing of extraction methods. The two best methods (The DNeasy Tissue kit and the MagNA Pure method) were chosen for use on patient samples. These methods possibly perform better because they contain few steps and because they do not contain any precipitation steps. A recent study comparing DNA extraction methods for extraction of *C. pneumoniae* DNA from vascular tissue also found a spin-column method (QIAamp DNA MiniKit) to give the best recovery [13]. Another study tested recovery percentages of five different spin-column DNA extraction methods for purification of viral DNA from serum samples. Here recovery percentages were also determined with quantitative PCR in the LightCycler and the QIAamp Blood kit (uses same principle as DNeasy and is manufactured by Qiagen) showed a relative recovery percentage of 76% [14] comparable to our absolute overall recovery percentage of 53.3%. and overall average relative recovery of 66.5%.

From Tables 1 and 2 it is apparent that there is considerable variation in how much DNA could be recovered even within methods (3.0–53.3% overall average recovery and 8.8–100% overall average relative recovery). Real-time quantitative PCR uses a point (the threshold cycle or crossing point) at the beginning of the log-phase of the PCR to determine the original target DNA concentration. This gives a more realistic, though not perfect picture, of the actual DNA concentration in the sample tested. The variation would not have been as apparent if PCR products had been analysed by gel electrophoresis [12,15] or time-resolved fluorometry (TRF) [6]. When comparing PCR bands in a gel or PCR product amounts by TRF they represent the end points of the PCRs (i.e. at the plateau phase of the PCR) and they are often very similar even for very different initial target DNA concentrations, especially if many PCR cycles are performed. Therefore, previous studies have probably not been fully aware of the actual variation in output of DNA from their extraction methods and therefore our results may seem highly variable in comparison. In the study comparing different spin-column methods for DNA purification, they found relative recovery percentages between 2.2–76% [14]. Therefore, it is apparent that even among methods based on similar principles there can be large differences in recovery of DNA [14].

Another reason for the apparent variability could be that the number determined by the real-time PCR depends not only on how much DNA is actually present, but also on the amount of inhibitors left in the sample, which will add further variation. It could be speculated that methods with low recovery percentages do not remove inhibitors sufficiently and are prone to DNA loss during purification, which in combination may give rise to the low recovery as measured by real-time PCR. In the early days of real-time PCR an estimation of the inhibition in the sample was obtained by calculating PCR efficiency for each sample using a dilution curve [16]. This was based on the assumption (and also other assumptions not mentioned here) that PCR efficiency is constant during the entire run, which stems from the PCR kinetics model currently used in the LightCycler software $R_n = R_0 * (1+E)^n$ (R_n: Fluorescence at cycle number n, R_0: initial amount of target, E: PCR efficiency). However, recent publications have indicated that this may not reflect the real PCR kinetics in a given sample as the efficiency varies form cycle to cycle and because other parameters in a sigmoidal model are probably better suited to describe the kinetics [17,18]. As it is still unclear how PCR kinetics is best described and how much trivial factors like variation in reaction components, thermal cycling conditions and mispriming events

contribute to deviations in PCR kinetics we have not incorporated PCR kinetics in the interpretation of our data. However, investigation of PCR kinetics under different conditions would clearly be helpful in selecting DNA extraction methods suitable for samples to be used in real-time PCR, as it possibly could be used to estimate the level of inhibition in the samples. This should obviously be a subject for future studies.

The rationale for using purified *C. pneumoniae* DNA in some of the samples was that input and output DNA concentrations were known or could be determined. However, this does not test whether the DNA extraction methods are capable of recovering DNA from whole EB. Therefore a test using tissue spiked with purified EB was also performed. It should be noted that this will not entirely reflect reality either as normally the EB are present inside human cells. Interestingly, the DNeasy Tissue kit has been used on lungs from mice experimentally infected with *C. pneumoniae* and here the kit was able to recover *C. pneumoniae* DNA amplifiable by real-time PCR [10]. Therefore, this kit is actually able to recover *C. pneumoniae* DNA from a real infection where the bacteria are present inside cells. Our study in combination with the study by Apfalter et al. [8] indicate that more work is needed to optimise DNA extraction methods. Automated DNA extraction methods like the MagNA Pure method used in this study may be the future choice, as these methods may be able to produce more reproducible results, but this remains to be examined more carefully.

Patient samples

The DNeasy Tissue kit and the MagNA Pure method were used on 78 atherosclerotic tissue samples and subsequently amounts of *C. pneumoniae* DNA and human DNA were determined with quantitative real-time PCR. We found no samples positive for *C. pneumoniae* DNA. All samples were positive for human DNA. As there was great variation in the amount of human DNA among samples, it could be suspected that low human DNA numbers are caused by inhibitors, as the tissue input in mg should be approximately equal for the samples. However, atherosclerotic tissue shows a very complex and variable composition which may contribute to the variability observed. Some tissue samples may contain high levels of calcium, which will decrease the amount of human DNA, or a high number of inflammatory cells, which will increase the amount of human DNA. The high variability in human DNA recovery from the patient samples can lead to the speculation that there may also be a high variability in recovery of *C. pneumoniae* DNA, especially as the bacterium is obligate intracellular. Therefore, as it has been proposed by a recent Australian study [19], it may be necessary to analyse more samples (up to 15 samples according to [19]) from different artery locations in each patient in order to obtain the true number of *C. pneumoniae* positive patients. This is according to this study [19] advantagous because the distribution of *C. pneumoniae* over the tissue is patchy and bacteria are present in low numbers. It should be noted though that using single random sections from 10 patients the Australian study found from 35.6–41.6% percent positives [19], whereas we find no positives in 78 patients using two samples purified with two different DNA extraction methods from the same site. Therefore, we will probably not observe as high numbers of positive patients even though more samples per patient were analysed. Especially in this experimental context where the PCR sensitivity is 2 genomes in the purified sample amount loaded directly into the PCR tube [9] and the sensitivity of the whole process of DNA extraction followed by PCR is less than 20 EB/μl (concentration in samples before DNA extraction).

It is highly probable that there are larger amounts of human DNA than of *C. pneumoniae* DNA present in the samples. Accordingly, inhibition will have a greater effect on the *C. pneumoniae* PCR results and consequently the human DNA PCR cannot be used as an inhibition control. Therefore, inhibition was tested in the samples by spiking them with two concentrations of *C. pneumoniae* DNA before PCR. Hereby, we found that the *C. pneumoniae* DNA could be detected in all samples. Therefore, it seems that the DNeasy Tissue kit and the MagNA Pure method did remove inhibitors sufficiently.

Short review of previously published studies on detection of C. pneumoniae in atherosclerotic tissue

To study whether there were methodological differences between studies with positive and negative results we compared all the negative studies with a selection of representative positive studies (Table 3). The positive studies were selected so that they represented studies both with high and low prevalence of C. pneumoniae and the entire span of years of C. pneumoniae direct detection in tissue by PCR. Furthermore, different countries were represented. Among the positive studies The QIAamp Tissue Kit and variations of phenol/chloroform extractions were mostly used. Most of the positive studies declare that they attempt to control contamination at some level, at least with reagent negative controls in the PCR runs. The early negative studies [25–27] only used Proteinase K as DNA extraction method, which may not be sufficient as, in most cases, it will leave behind many potential inhibitors. However, one of the positive studies did use Proteinase K only for extraction [32]. The Chelex [100 method used in one of the negative studies [7] may not be sufficient for purification either as it only removes multivalent cat ions and lyse cells [41]. The rest of the negative studies used DNA extraction methods optimised by their own laboratory [15] or methods also used by positive studies [6,20–

22]. Two negative studies used the modified version of the Easy-DNA kit [23,24]. This means that at least 7/11 negative studies used a DNA extraction method that should be suitable for purification of DNA from tissue. Four of these studies did also check sufficiently for PCR inhibition [6,21,22,24], but it is unclear whether the three other studies did [23,15,20]. Not surprisingly, some of the negative studies did use a DNA extraction method which might have been insufficient (4/11 negative studies). Three of these studies did check for inhibitors, by either addition of an internal processing control to the samples [7], spiking samples with C. pneumoniae DNA before PCR [26] or with a β-actin-specific PCR on the samples [25], however, as discussed above, testing for presence of human DNA may not be a sufficient inhibition control. This means that with regard to methodology, 9/11 negative studies did use sufficient DNA extraction methods or did check for inhibitors.

When comparing PCR methods used it is seen that almost the same number of negative and positive studies used a nested PCR assay (5/11 negative vs. 6/13 positive studies used nested PCR). The reason for higher positivity rates in studies using nested PCR could be either because nested PCRs are more sensitive or because they are more susceptible to contamination, but in the selected studies (table 3) there is no clear indication of this. A large number of positive studies used a PCR based on the *Pst*I fragment (1/10 negative vs. 6/13 positive studies used a *Pst*I-fragment based PCR). It could be speculated that either the *Pst*I fragment-based PCRs are either more prone to unspecific amplifications or they are more sensitive. This needs to be investigated in more detail.

Discussion of reasons for non-detection of C. pneumoniae *in Danish samples*

To date two Danish studies [6,7] and the present study have been conducted by three different Danish research units, and in no study has *C. pneumoniae* DNA been detected in atherosclerotic tissue. Negative results were obtained despite the use of optimised diagnostic tests and relatively large numbers of patient samples. The frequency of atherosclerosis in Denmark is not lower than in e.g. USA, England and Italy, where *C. pneumoniae* has been found in atherosclerotic tissue (Table 2) with high prevalence. These facts argue against a correlation of *C. pneumoniae* and cardiovascular disease.

Table 3: Publications with positive/negative results on direct detection by PCR of *C. pneumoniae* in atheroscle-rotic tissue

Authors	Year	PCR method	DNA purification	Samples collected in	PCR positive (%)
Wessely et al. [20]	2003	Semi-nested, 474-bp PstI fragment Nested, MOMP gene	phenol/chloroform	Germany	0/31 (0.0)
Vainio et al. [21]	2002	Nested, MOMP gene	QIAmp Tissue kit	Norway	0/48 (0.0)
Ong et al. [22]	2001	Nested, MOMP gene	QiAmp Tissue Kit	Northern Ire	0/44 (0.0)
Meijer et al. [23]	2000	Single-step, 16S rRNA and MOMP genes	Easy-DNA Kit, modified	The Netherlands	0/13 (0.0)
Meijer et al. [24]	1999	Single-step, 16S rRNA and MOMP genes	Easy-DNA Kit, modified	The Netherlands	0/18 (0.0)
Palfrey et al. [15]	1999	Single-step, MOMP gene	Gene Clean Kit	England	0/8 (0.0)
Lindholt et al. [6]	1998	Nested, MOMP gene	phenol/chloroform	Denmark	0/124 (0.0)
Andreasen et al. [7]	1998	Multiplex, Chlamydia species	Chelex 100	Denmark	0/22 (0.0)
Daus et al. [25]	1998	Semi-nested, 474-bp PstI fragment	Proteinase K	Germany	1*/29 (3.4)
Paterson et al. [26]	1998	Nested, omp1 gene	Proteinase K	Australia	0/49 (0.0)
Weiss et al. [27]	1996	Single-step, 16S rRNA gene	Proteinase K	Brooklyn, USA	0/58 (0.0)
Cochrane et al. [28]	2002	Single-step, 474-bp PstI fragment	Qiamp Tissue Kit	Australia	15/29 (51.7)
Valassina et al. [29]	2001	Single-step, omp1, 16S rDNA, Hsp70 gene	Qiamp Tissue Kit	Italy	13/58 (58.0)
Gutierrez et al. [30]	2001	Semi-nested, 474-bp PstI fragment	High Pure PCR Template preparation Kit	Spain	48/85 (56.5)
Farsak et al. [31]	2000	Single-step, 16S rDNA	phenol/chloroform +CTAB	Turkey	12/46 (26)
Jantos et al. [32]	1999	Single-step, omp1, 16S rDNA	Proteinase K	Germany	4/50 (8.0)
Wong et al. [33]	1999	Nested, omp1	phenol/chloroform	England	26/68 (38)
Petersen et al. [34]	1998	Single-step, omp1	Qiamp Tissue Kit	Sweden	14/40 (35.0)
Ouchi et al. [35]	1998	Nested, 474-bp PstI fragment	phenol/chloroform	Japan	16/29 (55.2)
Maass et al. [36]	1997	Nested, 474-bp PstI fragment	phenol/chloroform +CTAB	Germany	9/61 (14.8)
Ong et al. [37]	1996	Nested, MOMP gene	phenol/chloroform	England	19/43 (44.2)
Blasi et al. [38]	1996	Nested, 16S rDNA	phenol/chloroform	Italy	26/51 (51.0)
Campbell et al. [39]	1995	Single-step, 474-bp PstI fragment	phenol/Chloro form or Qiamp	USA	12/38 (31.6)
Kuo et al. [40]	1993	Single-step, 474-bp PstI fragment, 16S rDNA	Phenol/chloroform	South Africa	12/30 (40.0)

*The single positive specimen was not positive on retesting

A number of circumstances could explain why *C. pneumoniae* has not been demonstrated in atherosclerotic samples from Danish patients. From other studies it seems probable that there are great inter-laboratory differences in the handling of both the PCR and DNA extraction methods [8]. Several multicenter comparison trials have also shown that some laboratories may have contamination problems [8,42]. Consequently, it could be argued that high detection rates of *C. pneumoniae* DNA in atherosclerotic tissue, at least in part, may be caused by contamination. A recent study has for example shown that the nested PCR is highly unreliable for *C. pneumoniae* detection because it is very prone to contamination [43]. On the other hand it could be argued that the negative studies used insensitive or inappropriate techniques. However, the present study and most other studies with negative results have attempted to optimise methods to ensure optimal sample preparation or inhibition control and they used a sensitive PCR technique. However, as discussed above it may be necessary to optimise methods further and to include more samples from each patient [19].

It is possible that divergence between studies is caused by periodical presence of *C. pneumoniae* in blood vessels dependent on the local epidemic conditions and *C. pneumoniae* is just transiently present during epidemics. The *C. pneumoniae* seroprevalence is not as high in Denmark as in many other countries where seroprevalence is often over 50%. Among Danish blood donors without respiratory tract symptoms it was approximately 40% [44]. In Denmark there has apparently not been a *C. pneumoniae* epidemic since the early eighties [45]. One study found 1.3% (28/ 2219 samples) prevalence of *C. pneumoniae* by culture and PCR in Danish respiratory tract samples sent to the Chlamydia laboratory at Statens Serum Institut in Denmark from 1993 to 1995. (Most routine detection of *C. pneumoniae* in Denmark is performed at Statens Serum Institut) [46]. This low prevalence of *C. pneumoniae* in respiratory tract samples may explain why *C. pneumoniae* DNA is not found in the Danish atherosclerotic tissue as the vascular *C. pneumoniae* infections are likely to originate from the respiratory tract infections. In table 3 it is seen that the prevalence of *C. pneumoniae* in atherosclerotic tissue from different countries varies between 0–58%, which indicates variations that may be caused by local epidemic conditions, even though not all positive studies published are represented in the table. However, no countries at present maintain sentinel surveillance of *C. pneumoniae* infections and results from local health authorities and research reports have not yet provided evidence that different epidemic conditions could cause the differences.

Conclusions

In conclusion we found that: 1) The DNeasy Tissue kit and the MagNA Pure method were the methods with the highest recoveries when five DNA extraction methods were tested on four different sample panels. Therefore, in our hands, these two methods are most suitable for purification of DNA from atherosclerotic tissue, when using our LightCycler real-time PCR for *C. pneumoniae* DNA quantification in the samples. 2) DNA was purified from 78 atherosclerotic tissue samples with both methods. Danes in this study did not seem to harbor *C. pneumoniae* in atherosclerotic tissue, which confirms previous Danish studies. However, the fact that human DNA levels varied could indicate that the likelyhood of detecting *C. pneumoniae* vary despite optimisation and test of the methods. 3) Inhibitor levels were seemingly low in the purified DNA from the patients samples, as *C. pneumoniae* DNA used for spiking of the 2 × 78 DNA preparations from the patient samples could be refound in them all. 4) Further testing of DNA extraction methods is needed as this study has shown considerable intra- and inter-method variation in DNA recovery. Future studies could focus on using real-time PCR for investigation of the PCR kinetics in samples purified with different DNA extraction methods as this might more accurately reflect the amount of PCR inhibitors left in the samples.

Methods

Preparation of C. pneumoniae *genomic DNA*

C. pneumoniae was cultivated and EB purified as described previously [47]. *C. pneumoniae* DNA was purified from EB with the Qiagen Blood and Cell culture DNA Midi Kit as instructed by the manufacturer and concentration was measured with a spectrophotometer as previously described [9,10].

Sample panels

Atherosclerotic tissue homogenates samples spiked with C. pneumoniae *DNA*

2.5 g of human aorta tissue was homogenised in TE-buffer with a hand-held homogenisator with rotating knives. The volume of the homogenate was increased to 25 ml with TE-buffer and divided into 5 ml portions. Three portions were spiked with purified *C. pneumoniae* DNA of known concentration. The concentrations in the homogenate were after spiking: 10^5 copies/μl, 10^3 copies/μl, 10 copies/μl. Copies/μl refer to genome copies per microliter. One portion was left unspiked as a negative control. The four 5 ml portions were divided into 100 μl aliquots so that each aliquot corresponded to approximately 10 mg of tissue. Spiked homogenates were frozen in aliqouts of 100 μl at -70°C and each time a DNA purification method was tested two aliquots for each spiking concentration and the negative control were thawed and extracted.

Pure DNA samples

For each DNA purification method a dilution series of *C. pneumoniae* DNA with concentrations 10^5 copies/μl, 10^3 copies/μl, 10 copies/μl and a negative control without *C. pneumoniae* DNA was also included. Dilutions were made in double distilled water with 20 μg/ml yeast RNA as a carrier nucleic acid. Pure DNA samples were frozen in aliquots of 100 μl at -70°C and each time a DNA purification method was tested two aliquots for each DNA concentration and the negative control were thawed and extracted.

Purified EB samples

For each DNA purification method a dilution series of purified intact *C. pneumoniae* EB and a negative control was tested. The EB preparation was examined by electron microscopy and found to consist of intact EB and very limited amounts of intermediate forms between EB and RB. To obtain an estimate of the number of EB in the purified EB preparation a test purification was performed with the DNeasy Tissue kit and number of output *C. pneumoniae* genomes was quantified with the *C. pneumoniae* real-time PCR as described below. The concentration in the EB preparation was estimated to be 2.4×10^7 EB/μl. Based on the estimate of EB concentration a dilution series of the EB was prepared so that the last dilution would yield DNA concentrations at or below the detection limit of the *C. pneumoniae* real-time PCR. Therefore EB were diluted in PBS to 1.2×10^{-4}, 1.2×10^{-5}, 1.2×10^{-6}, 1.2×10^{-8} of the original EB concentration (corresponding to approximate EB concentrations of 2000, 200, 20, 0.2 and 0 EB/μl). Samples were frozen in aliquots of 100 μl at -70°C and each time a DNA purification method was tested two aliquots for each DNA concentration and the negative control were thawed and extracted.

Atherosclerotic tissue homogenates samples spiked with C. pneumoniae *EB*

2.5 g of human aorta tissue was homogenised in TE-buffer with a hand-held homogenisator with rotating knives. The volume of the homogenate was increased to 25 ml with TE-buffer and divided into 5 ml portions. Four portions were spiked with purified *C. pneumoniae* EB of known concentration and one left unspiked as a negative control. The EB concentrations were the same as in the purified EB samples. The five 5 ml portions were divided into 100 μl aliquots so that each aliquot corresponded to approximately 10 mg of tissue. Spiked homogenates were frozen in aliqouts of 100 μl at -70°C and each time a DNA purification method was tested two aliquots for each spiking concentration and the negative control were thawed and extracted.

DNA extraction methods

Phenol/chloroform extraction

To the 100 μl sample was added lysis-buffer (30 mM tris-HCl pH 8.0, 30 mM EDTA, 150 mM NaCl, 0.5% SDS, 0.5% Triton X-100, 0.5% Tween 20) up to 250 μl. 125 μg Proteinase K was added and the samples were incubated at 55°C until the tissue was completely digested. 5 μg RNase was added and the samples incubated 1/2 hour at 37°C. Samples were extracted with phenol/chloroform/isoamylethanol (25:24:1), the DNA was precipitated with 96% ethanol and 3 M sodium acetate pH 5.2, washed in 70% ethanol and dried in a vacuum centrifuge. The DNA pellet was dissolved in 100 μl Tris-EDTA pH 8.0.

Phenol/chloroform extraction with Cetyltrimethylammonium bromide (CTAB) precipitation

This method was performed as described above, but with two additional steps. After the RNase treatment 42 μl 5 M NaCl and 33 μl 10% CTAB was added to each sample and the samples were incubated at 65°C for 10 min. Samples were centrifuged and the supernatant was transferred to new tubes.

DNeasy Tissue Kit (Qiagen)

Instructions from the manufacturer were followed. Briefly, the method consists of the following steps. The 100 μl samples were lysed with Qiagen Proteinase K and lysis buffer, loaded onto DNeasy spin columns with a silica-gel membrane (DNA-binding), washed and eluted in 100 μl elution buffer.

Easy-DNA Kit (Invitrogen)

Instructions from the manufacturer were followed. Briefly, the 100 μl sample was lysed with solution A, precipitated with solution B, and chloroform extracted. DNA was precipitated with ethanol and the washed and dried pellet was resuspended in 100 μl Tris-EDTA pH 8.0.

MagNA Pure LC DNA Isolation Kit III (Roche)

Instructions from the manufacturer were followed. Briefly, the 100 μl samples were lysed with Bacterial Lysis Buffer and Proteinase K for 10 min. at 65°C. Samples were then boiled for 10 min. at 95°C to inactivate microorganisms and transferred to the MagNA Pure LC instrument. This instrument handles all subsequent pipetting and magnetic isolation. A buffer containing a chaotrophic salt and Magnetic Glass Particles was added. DNA binds to the Magnetic Glass Particles and they are recovered with a magnet. Unbound molecules are removed by several washing steps and DNA is eluted in 100 μl elution buffer.

C. pneumoniae *DNA real-time PCR*

The real-time PCR was performed on 2 μl of the purified samples in a total PCR volume of 20 μl as described earlier [9] with addition of 1.5 U heat-labile Uracil-DNA

Glycosylase (Roche, Denmark) to ensure that amplicon contamination was not present. Briefly, a standard dilution series of purified genomic *C. pneumoniae* DNA with concentrations 10^5, 10^4, 10^3, 10^2, 10, 5, 1 copies/μl were used for quantification, here 2 μl was also added to a total PCR volume of 20 μl. The standard dilution series and a reagent negative control were included in all PCR runs. The lowest concentration (1 copy/μl) can be detected in approximately 70% of PCR runs [9]. As 2 μl is added per reaction it means that 2 copies of the genome per reaction can be detected in 70% of experiments. Therefore, the real-time PCR is highly sensitive. Primers amplified a 140 bp fragment from the *pmp4* gene and it was detected with a set of fluorescence resonance energy transfer (FRET) probes also specific for the *pmp4* gene in a Lightcycler instrument (Roche). The *pmp4* gene is a single copy gene and it belongs to the *pmp* gene family, which encodes the polymorphic membrane proteins. PCR was performed with the enzyme mix Faststart DNA Master Hybridization Probes (Roche) as described [9], except that incubation at room temperature was added before starting the cycling program to allow the Uracil-DNA Glycosylase to work. For each unknown sample the threshold cycle was measured for two replicates, and the DNA concentrations were calculated using the Lightcycler software, and the mean concentration for each sample was used for analysis.

Human DNA real-time PCR

The number of human genomes in the patient samples was determined by quantitative real-time PCR. Identical primers can be used for both the mouse and the human *gap* genes [10]. Primers amplified a 155 bp amplicon, which was detected with a FRET probe set. Lightcycler PCR was performed as described earlier except that the standard dilution series was 10^4, 10^3, 10^2, 10, 5, 1 copies/μl of human genomic DNA. Human genomic DNA was purified from Hep-2 cells with the Qiagen Blood and Cell culture DNA Midi Kit following descriptions from the manufacturer and DNA concentration was determined by measuring OD at 260 nm as previously described [9,10]. *Mycoplasma mycoides* PG3 DNA was used as carrier nucleic acid at a concentration of 10 μg/ml.

Precautions to avoid contamination

To avoid contamination we took a number of precautions. 1) DNA preparation, PCR preparation and PCR were performed in physically separated rooms. 2) Filter pipette tips were used for all pipettings. 3) PCR was performed with the Faststart DNA Master Hybridisation Probes kit, which contains dUTP instead of dTTP; this gives the opportunity to remove amplicon contamination with Uracil DNA Glycosylase. 4) The Lightcycler operates with a closed-tube system, where it is not necessary to open tubes after a PCR run, this minimises contamination especially with amplicon. 5) If tubes were broken by accident, the contaminated equipment was immediately decontaminated with DNAZap from Ambion. 6) In every PCR run at least one reagent negative control was included. 7) Extractions of *C. pneumoniae* DNA negative samples were included for each DNA purification to ensure that contamination was not introduced during purification.

Test of the five DNA extraction methods

All DNA extraction methods were tested on 100 μl of the four sample types; 1) pure *C. pneumoniae* DNA samples with concentrations 10^5, 10^3, 10 and 0 copies/μl, 2) atherosclerotic tissue homogenate spiked with pure *C. pneumoniae* DNA so that concentrations were 10^5, 10^3, 10 and 0 copies/μl in the spiked samples. 3) Purified *C. pneumoniae* EB in dilutions 1.2×10^{-4}, 1.2×10^{-5}, 1.2×10^{-6}, 1.2×10^{-8} and a negative control containing PBS only (corresponding to approximate EB concentrations of 2000, 200, 20, 0.2 and 0 EB/μl). 4) atherosclerotic tissue homogenate spiked with purified *C. pneumoniae* EB in dilutions 1.2×10^{-4}, 1.2×10^{-5}, 1.2×10^{-6}, 1.2×10^{-8} and a negative control (corresponding to approximate EB concentrations of 2000, 200, 20, 0.2 and 0 EB/μl). As end point for all methods samples were dissolved or eluted in a 100 μl volume. For each DNA purification method two replicates of each concentration and from each sample type (i.e. 26 samples per method) were purified. Subsequently, copies/μl of *C. pneumoniae* genomic DNA were determined for 2 μl of each sample by real-time PCR in the Lightcycler. Recovery percentages were calculated (Table 1) for each concentration of pure DNA samples and spiked aorta samples. For the purified EB samples output concentrations were compared relatively as the exact input EB concentration was not known.

Patient samples

Samples were obtained from Caucasian Danes admitted to the Unit of Vascular Surgery, Skejby Sygehus, Aarhus University Hospital, Denmark in the period 1994 to 1998. All patients underwent arterial repair due to acute or chronic atherosclerotic disease. During the procedure tissue was freshly and immediately frozen at -70° C at which temperature it was stored until analysis. Of the 78 samples, 25 samples were obtained from 23 women with a median age of 68 years (range: 48 to 83 years) and 53 samples were obtained from 50 men with a median age of 65 years (42 to 85 years). The samples were obtained from the following anatomical sites; aorta (5), carotid artery (23), iliac artery (7), femoral artery (38), and popliteal artery (3).

Purification of DNA from patient samples, real-time PCR quantification and inhibition test

DNA was purified with the DNeasy Tissue kit and the MagNA Pure method. This kit has a maximum tissue

input of 25 mg. To attempt to even out differences in different regions of the tissue samples, approximately 100 mg of tissue from each sample was cut in small pieces with a scalpel. An aliquot corresponding to 25 mg was subsequently used for purification with the DNeasy Tissue kit/ MagNA Pure method and DNA was eluted in 100 µl. *C. pneumoniae* genomic DNA concentration and human genomic DNA concentration were determined using 2 µl of sample with the Lightcycler real-time PCR quantitative assays described above. For both PCR assays concentrations were determined for two replicates of each of the 78 samples and average concentration was calculated. All samples were tested for inhibition by spiking with two different concentrations of *C. pneumoniae* genomic DNA. Ten µl clinical sample was spiked with either 10 µl of 10^3 copies/µl or 10 copies/µl of *C. pneumoniae* genomic DNA. These relatively large spike volumes were used in order to reduce uncertainties introduced by pipetting. For real-time quantitative PCR was used 4 µl of the spiked sample in a 20 µl total PCR volume. Four µl was used in the PCR instead of 2 µl to compensate for the dilution of the samples done during the spiking process.

Authors' contributions

TM performed the real-time PCRs, data analysis and drafted the paper. JSL collected the clinical samples and provided the clinical data. TM, LØ, SB and GC participated in the design and coordination of the study and editing of the manuscript.

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