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ORIGINAL ARTICLE

A comparison of methods for extracting DNA from *Coxiella burnetii* as measured by a duplex qPCR assay

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Abstract

Aims: To determine the optimal DNA extraction method for the detection of *Coxiella burnetii* including the small-cell variant (SCV) by real-time PCR (qPCR) in clinical samples.

Methods and Results: A duplex qPCR detecting two *Coxiella burnetii* gene targets (*com1* and *IS1111a* genes) was developed. Each target in this PCR had a sensitivity of one copy number per reaction. DNA extraction methods were compared on spiked negative samples and included a silica column kit, a chloroform separation prior to a silica column method and a chloroform/phenol separation and DNA precipitation method.

Conclusions: The silica column extraction method was more efficient at recovering *C. burnetii* DNA, from large-cell and small-cell variants, than a chloroform or chloroform/phenol method. The silica column method was useful on spiked human samples including serum, buffy coat and bone marrow samples.

Significance and impact of study: This study demonstrated that a simple column kit method is efficient to use for the detection of *C. burnetii* in clinical samples including the SCV.

Introduction

Coxiella burnetii is the intracellular bacterium that causes Q fever. Diagnosis of Q fever is generally made serologically with the gold standard immunofluorescence assay. Other diagnostic methods include enzyme-linked immunoassay, complement fixation (Field *et al.* 2000), polymerase chain reaction (PCR) (Fournier and Raoult 2003) and cell culture (Musso and Raoult 1995). PCR is a quick method that allows for diagnosis in the early phase of the disease (Fournier and Raoult 2003) but requires efficiency in extraction of bacterial DNA for diagnostic sensitivity.

Some sample types contain substances that inhibit or reduce PCR amplification efficiency (Akane *et al.* 1994). If these inhibitors were copurified with the microbial DNA prior to analysis, this would contribute to false negative test results. Serum samples have been used for the molecular detection of *C. burnetii* (Fournier and Raoult 2003). Serum contains fewer inhibitors than other samples such as blood, but does contain some inhibitors, including immunoglobulin G (IgG) (Al-Soud *et al.* 2000). Serum samples are a less preferable specimen than peripheral blood mononuclear cells as it is assumed that most of the *C. burnetii* would be found intracellularly within the buffy coat fraction. However, diagnostic blood specimens contain PCR inhibitors such as haemoglobin (Akane *et al.* 1994), lactoferrin (in leucocytes; Al-Soud and Radstrom 2001) and anticoagulants such as EDTA (Wang *et al.* 1992) or heparin (Yokota *et al.* 1999), which may confound testing of this sample type.

Previous studies have shown that in chronic Q fever, *C. burnetii* DNA is present in bone marrow samples (Peacock *et al.* 1983) in which it can persist for up to 12 years after primary infection (Harris *et al.* 2000; Marmion *et al.* 2005). Bone marrow has similar inhibitors as blood with an increased amount of host (eukaryotic) DNA, which is itself inhibitory to PCR (Roussel *et al.* 2005).

Coxiella burnetii in bone marrow is postulated to be in the small-cell variant (SCV) form, which may be more

resistant to lysis during the DNA extraction process, leading to false negative test results (Marmion *et al.* 2005). Samples containing this cell type may require more vigorous lysis to ensure the complete extraction of *C. burnetii* DNA.

In this study, three different methods of DNA extraction (with variations) were compared for the detection of *C. burnetii* in a variety of clinical samples. These samples were spiked with *C. burnetii* cells from cell cultures including those grown under conditions to increase the amount of cells in the SCV form. Two *C. burnetii*-specific qPCRs targeting the *com1* gene and a bacterial insertion sequence were combined in a duplex qPCR and were analysed for their sensitivity and specificity. This qPCR was then used to measure DNA extracted by the different methods.

Materials and methods

Positive and negative controls

Positive DNA templates for qPCR quantification. The amplicons produced by *com1* and *IS1111a* PCR of *C. burnetii* (Nine Mile clone 4 RSA-439) were cloned using the TOPO TA cloning kit (Invitrogen, Mulgrave, Australia) with the Top10 *E. coli* cells (Invitrogen) following the manufacturer's instructions. The resulting plasmids were purified using the Plasmid Maxi Kit (Qiagen, Hilden, Germany) as per the manufacturer's specifications. The purified plasmids were diluted 1 : 100 and the theoretical copy numbers quantified from the OD_{260} reading measured by Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Fremont, CA, USA). A series of 1 : 10 serial dilutions of the purified plasmid was analysed by qPCR (in triplicate) to create a standard curve from which the sensitivity of each reaction could be determined and from which the amount of DNA ($\mu g\ \mu l^{-1}$) in the final eluate of the spiked samples (extracted by various methods) was calculated.

Positive DNA templates for spiking. DNA extracted by the silica column method (48-silica with a digestion time of 10 min) from *C. burnetii* (Nine Mile clone 4 RSA-439) was used as a positive control for all PCRs. This extracted DNA was also used to spike samples post-DNA extraction to determine if any PCR inhibitors were present in the final eluate.

Positive C. burnetii *cell culture for spiking. Coxiella burnetii* (Nine Mile clone 4) grown in Vero cells at 35°C with 5% CO_2 (Sanyo, Moriguchi, Japan) with Roswell Park Memorial Institute 1640 media (Gibco Invitrogen) supplemented with 200 mmol l^{-1} of L-glutamine (Gibco) and 3–10% newborn calf serum (Gibco). As the Nine Mile clone 4 isolate of *C. burnetii* is an exception to the level 3 pathogen status of other *C. burnetii* isolates, this was done under physical containment (PC) level 2 (American CDC select agents exclusions available at: http://www.selectagents.gov/Select%20Agents%20and%20Toxins%20Exclusions.html accessed 9 February 2011). *C. burnetii* grown in these conditions was used to spike samples prior to DNA extraction to determine the efficiency of the methods of extraction. In some cases, cultures that had been induced to produce an increased proportion of SCV by a method described previously (Coleman *et al.* 2004) were used to determine the efficiency of DNA extraction from these cell types. Some of these SCV-enriched cultures were also passed through a 0·22-μm filter to reduce the number of large-cell variants (LCV). For comparative purposes, Q-fever Vaccine Q-Vax® (CSL, Melbourne, Australia) which contains formalin-fixed whole *C. burnetii* (Henzerling isolate) was also used (both 0·22-μm filtered and unfiltered) to spike bone marrow samples prior to DNA extraction.

Negative DNA templates. To determine the specificity of the PCR, DNA was extracted from a selection of other bacteria (*Anaplasma phagocytophilum*, *Bacillus cereus, Capnocytophaga canimorsus*, *Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Legionella pneumophila, Listeria monocytogenes, Moraxella catarrhalis, Proteus mirabilis, Pseudomonas aeruginosa, Rickettsia australis*, *Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus pneumoniae*). These bacteria were isolated and identified by a pathology laboratory and were not formal type strains. Eluted DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific), the OD_{260} reading measured was converted to copy numbers and the DNA diluted to a concentration containing approximately 1000 copies per qPCR reaction.

DNA extraction from spiked human blood

Different methods of DNA extraction were used in this study and are described below. The names and details of the methods used are also given in Table 1.

Method RBC-silica: DNA was extracted from 200 μl of 'buffy coat' (concentrated peripheral blood mononuclear cells), plasma or serum. Buffy coat samples were first separated from whole blood samples prior to DNA extraction using red blood cell lysis (RBCL) buffer (Gentra Systems, Minneapolis, MN, USA) as per the manufacturer's instructions and resuspended in 600 μl of sterile PBS (Oxoid, Cambridge, UK). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. The amount of elution buffer (AE) (Qiagen) added to the column was reduced to 50 μl to provide a more concentrated DNA eluate.

Table 1 Methods of DNA extraction used

Method name	Sample type used for	Proteinase K digestion	RBC lysis usage	Silica column usage	Chloroform usage	Phenol usage
RBC-silica	Blood	10 min	Yes	Yes	No	No
48-silica	BM	48 h	No	Yes	No	No
RBC-48-silica	BM	48 h	Yes	Yes	No	No
C-silica	BM	48 h	No	Yes	Yes	No
95-C-silica	BM	48 h	No	Yes	Yes	No
PHC	BM	No	No	No	Yes	Yes

BM, bone marrow.

DNA extraction from spiked human bone marrow

Methods 48-silica and RBC-48-silica: DNA was extracted from bone marrow samples using the QIAamp DNA Mini Kit according to the manufacturer's protocol, with an increased digestion time of 48 h at 56°C to ensure complete lysis of the bone marrow (48-silica). A variation of this method involved a pretreatment with RBCL buffer as per the manufacturer's instructions and resuspended in 600 μl of sterile PBS followed by the QIAamp DNA Mini Kit column extraction (RBC-48-silica).

Methods C-silica and 95-C-silica: Method C-silica involved chloroform extraction followed by column extraction as described previously (Marmion *et al.* 2005). A variation of this method included an additional heating step, 95°C for 15 min, following the addition of the TE buffer prior to column extraction (95-C-silica).

Method PHC: A phenol–chloroform extraction method was also used. In a 1·5-ml tube, 200 μl of sample was added to 200 μl of phenol:chloroform (1 : 1 v/v) and vortexed for 5-s. The sample was then spun at 14 000 ***g*** for 2 min. The aqueous layer was removed into a sterile 1·5-ml tube. Phenol/chloroform (200 μl) was added, and the mixing, centrifuging and separation of the aqueous phase were repeated. Chloroform (200 μl) was added to the second separated aqueous phase, and the mixing, centrifuging and separation of the aqueous phase were repeated once more. The final aqueous phase was ethanol precipitated, and the resulting pellet was resuspended in 50 μl of elution buffer (AE buffer from the QIAamp DNA Mini Kit).

qPCR analysis (real-time quantitative PCR)

Primers and probe targeting a highly conserved 27-kDa outer membrane protein (*com1* gene) and a second set targeting the bacterial insertion sequence *IS1111a* were used. The primers amplifying 76 bp of the *com1* gene and probe were designed by Paul Storm (personal communication) and designated com1F (5′-AAA ACC TCC GCG TTG TCT TCA-3′) com1R (5′-GCT AAT GAT ACT TTG GCA GCG TAT TG-3′) and com1probe (5′-FAM-AGA ACT GCC CAT TTT TGG CGG CCA-BHQ1-3′) (Biosearch Technologies Inc., Novato, CA, USA). These primers and probe had 100% homology with all five *C. burnetii* genomes completely sequenced (CP001020·1, CP001019·1, CP000890·1, CP000733·1 and AE016828·2) by blast search. The primers and probe amplifying 85 bp of the insertion sequence (*IS1111a*) were described previously (Banazis *et al.* 2010). Each reaction contained 400 nmol l^{-1} of *com1* primers, 200 nmol l^{-1} of *com1* probe and *IS1111a* primers and 100 nmol l^{-1} of *IS1111a* probe. Oligonucleotides with Platinum qPCR supermix-UDG Master Mix (Invitrogen) supplemented with 0·75 μl of 1·5 mmol l^{-1} MgCl and 5 μl of extracted DNA were combined with nuclease-free H_2O to a final reaction volume of 25 μl. These assays could be used separately or together in a duplex assay. For each reaction, one positive *C. burnetii* control was used with a negative 'no template control' and a negative 'extraction control'. The qPCR was performed in a Rotor-Gene 3000 thermocycler (Qiagen, Doncaster, Australia) with an initial holding temperature of 50 °C for three min, followed by 95 °C for five min and then 60 cycles of 95 °C for 20 s and 60 °C for 40 s.

Statistical analysis

Inhibition of the PCR was considered to be a significant reduction in the amount of DNA detected. Each DNA extraction was performed in triplicate, and the significance between qPCR results was determined by the Student's *t*-test comparing the estimated concentrations of *C. burnetii* DNA (in $\mu g\ \mu l^{-1}$) detected by the *com1* assay.

Results

PCR reaction

The sensitivity of both qPCR reactions was determined to be approximately one copy of the target gene by standard

curves produced by analysis of the 1 : 10 serial dilutions of the cloned plasmids (data not shown). No reduction in sensitivity was observed when the two reactions were combined into a duplex assay. All other intracellular bacteria and medically important bacteria tested were negative for both targets. The *com1* qPCR result was used to estimate the concentration of *C. burnetii* DNA (in $\mu g\ \mu l^{-1}$) in each sample.

Spiked blood samples

DNA was extracted by the silica column (RBC-silica) from negative clinical samples (buffy coat, plasma and serum) spiked with *C. burnetii* and compared to a spiked PBS control. Higher estimates of *C. burnetii* DNA concentration were observed for the spiked buffy coat (22·6 ± 0·8 $\mu g\ \mu l^{-1}$), plasma (22·0 ± 1·6 $\mu g\ \mu l^{-1}$) and serum (22·6 ± 2·9 $\mu g\ \mu l^{-1}$) samples than in the PBS control (17·6 ± 1·7 $\mu g\ \mu l^{-1}$), indicating that there was no inhibition of the qPCR in these spiked mock clinical samples. These differences were not significant.

Inhibition of the qPCR by an excess of eukaryotic DNA in buffy coat samples was further investigated. Previously, extracted DNA from 88 *C. burnetii* negative buffy coat specimens was spiked with *C. burnetii* DNA and compared with matched PBS controls. No inhibition of the qPCR reaction was observed in these specimens.

DNA extraction from the SCV-enriched cultures of *C. burnetii*

Three methods (RBC-silica, 48-silica and RBC-48-silica) were compared for the extraction of DNA from SCV-enriched cultures. More DNA was extracted by method RBC-silica (16·6 ± 4·4 $\mu g\ \mu l^{-1}$) and method 48-silica (33·9 ± 3·6 $\mu g\ \mu l^{-1}$) than by method 95-C-silica (0·2 ± 3·3 $\times 10^{-2}$ $\mu g\ \mu l^{-1}$) ($P = 0{\cdot}003$ and $P = 0{\cdot}0005$ respectively). The increase in the amount of extractable DNA resulting from the 48-h digestion (48-silica) when compared to the normal 10 min digestion did not reach statistical significance ($P = 0{\cdot}07$), suggesting that lysis of the SCV was time dependent.

DNA extraction from bone marrow spiked with filtered *C. burnetii*

Bone marrow samples were spiked with either A) an unfiltered *C. burnetii* cell culture preparation (containing a mixture of LCV and SCV but enriched for SCV) or B) a 0·22-μm filtered *C. burnetii* cell culture preparation (enriched for SCV) of *C. burnetii* cultures in Vero cells under conditions that favour formation of SCV. These preparations were used to compare the extraction of *C. burnetii* DNA by four different extraction methods (Table 2).

The column extraction (48-silica) recovered large amounts of DNA from both unfiltered and filtered *C. burnetii*-spiked samples. The addition of a heating step (95-C-silica) in the chloroform method significantly reduced the amount of detectable DNA from samples spiked with a mixture of LCV and SCV compared with those extracted by the chloroform method (C-silica; $P = 0{\cdot}0024$). The silica column method (48-silica) also extracted significantly more detectable DNA than the chloroform method with the addition of the heating step (95-C-silica) ($P = 0{\cdot}014$). This difference was not seen in

Table 2 *Coxiella burnetii* DNA (qPCR Ct values, copy numbers and $\mu g\ \mu l^{-1}$) extracted by various methods from 'spiked' human bone marrow samples

	Unfiltered *C. burnetii*			Filtered *C. burnetii*		
Method	qPCR Ct value	Theoretical copy numbers	$\mu g\ \mu l^{-1}$	qPCR Ct value	Theoretical copy numbers	$\mu g\ \mu l^{-1}$
SCV enriched culture						
48-silica	14·7 ± 0·1	$2{\cdot}4 \times 10^{6} \pm 1{\cdot}5 \times 10^{5}$	5·4 ± 0·3	23·47 ± 0·59	$5 \times 10^{3} \pm 2{\cdot}3 \times 10^{3}$	$1{\cdot}1 \times 10^{-2} \pm 5{\cdot}1 \times 10^{-3}$
C-silica	14·3 ± 0·2	$3{\cdot}1 \times 10^{6} \pm 3{\cdot}3 \times 10^{5}$	6·8 ± 0·7	24·7 ± 0·4	$2{\cdot}1 \times 10^{3} \pm 5{\cdot}5 \times 10^{2}$	$4{\cdot}7 \times 10^{-3} \pm 1{\cdot}2 \times 10^{-3}$
95-C-silica	16·4 ± 0·3	$6{\cdot}9 \times 10^{5} \pm 1{\cdot}6 \times 10^{5}$	1·5 ± 0·4	24·4 ± 0·6	$2{\cdot}7 \times 10^{3} \pm 1{\cdot}0 \times 10^{2}$	$5{\cdot}9 \times 10^{-3} \pm 2{\cdot}2 \times 10^{-3}$
PHC	16·4 ± 1·0	$8{\cdot}2 \times 10^{5} \pm 5{\cdot}5 \times 10^{5}$	1·8 ± 1·2	27·8 ± 1·7	$3{\cdot}1 \times 10^{2} \pm 2{\cdot}4 \times 10^{2}$	$7{\cdot}0 \times 10^{-4} \pm 5{\cdot}2 \times 10^{-4}$
Q-Vax®						
48-silica	15·5 ± 0·4	$1{\cdot}4 \times 10^{6} \pm 3{\cdot}7 \times 10^{5}$	3·1 ± 0·8	20·7 ± 0·1	$3{\cdot}3 \times 10^{4} \pm 2{\cdot}2 \times 10^{3}$	$7{\cdot}4 \times 10^{-2} \pm 5{\cdot}0 \times 10^{-3}$
RBC-48-silica	16·3 ± 1·1	$8{\cdot}5 \times 10^{5} \pm 5{\cdot}1 \times 10^{5}$	1·9 ± 1·3	21·3 ± 0·4	$2{\cdot}4 \times 10^{4} \pm 6{\cdot}5 \times 10^{3}$	$5{\cdot}2 \times 10^{-2} \pm 1{\cdot}5 \times 10^{-2}$
C-silica	15·9 ± 0·1	$1{\cdot}0 \times 10^{6} \pm 9{\cdot}0 \times 10^{4}$	2·2 ± 0·2	24·3 ± 0·7	$2{\cdot}8 \times 10^{3} \pm 1{\cdot}2 \times 10^{3}$	$6{\cdot}2 \times 10^{-3} \pm 2{\cdot}7 \times 10^{-3}$
95-C-silica	15·6 ± 0·4	$1{\cdot}2 \times 10^{6} \pm 4{\cdot}1 \times 10^{5}$	2·8 ± 0·9	24·9 ± 0·7	$1{\cdot}8 \times 10^{3} \pm 9{\cdot}4 \times 10^{2}$	$4{\cdot}1 \times 10^{-3} \pm 2{\cdot}1 \times 10^{-3}$

SCV, small-cell variant.

DNA extraction methods are described in Table 1. ±1 SD is given. Unfiltered *C. burnetii* contained large-cell variants and small-cell variants. Filtered *C. burnetii* (0·22 μm) was presumed to contain small-cell variants only. Theoretical copy numbers and $\mu g\ \mu l^{-1}$ were calculated from the qPCR Ct value.

samples spiked with 0·22-μm filtered (SCV) *C. burnetii*. Samples spiked with 0·22-μm filtered cultures and extracted by the chloroform method with heating (95-C-silica) had significantly higher detectable DNA concentrations than those extracted by the phenol–chloroform method (PHC; $P = 0{\cdot}032$). Those extracted by the silica column method (48-silica) had significantly more detectable DNA than those extracted by the chloroform method (C-silica; $P = 0{\cdot}028$) and the phenol–chloroform method (PHC; $P = 0{\cdot}044$). The phenol–chloroform method (PHC) yielded only small amounts of detectable DNA with both spiked sample sets.

This extraction comparison was repeated using the Q-fever vaccine Q-Vax® in place of the *C. burnetii* Vero cell culture. The methods compared were 48-silica, RBC-48-silica, C-silica and 95-C-silica. Results are also shown in Table 2. There was no significant difference in the amount of DNA detected between the four extraction methods in samples spiked with unfiltered Q-Vax®. However, with those spiked with filtered Q-Vax® (SCV), there was significantly more DNA detected in samples extracted by the column method (48-silica) compared to the chloroform method (C-silica $P = 0{\cdot}02$ or 95-C-silica $P = 0{\cdot}01$). There was significantly more DNA detected in samples extracted by the silica column method with RBC lysis (RBC-48-silica) when compared to the chloroform method (C-silica $P = 0{\cdot}04$ or 95-C-silica $P = 0{\cdot}02$).

The silica column method was more efficient than the other methods for DNA extraction from *C. burnetii*. To determine if a reduction in the amount of DNA detected was because of carry over of PCR inhibitors, samples were spiked with extracted *C. burnetii* DNA after DNA extraction and were compared to a matched PBS control. No inhibition was found (data not shown).

Discussion

This study was undertaken to establish the optimal method of DNA extraction from *C. burnetii* and to use a newly developed qPCR for detection of *C. burnetii* DNA in spiked human samples.

The *com1* and *IS1111a* duplex qPCR assay was highly specific, producing negative results for all other bacteria tested. Both assays were highly sensitive, detecting one copy per reaction as a single assay and when combined in a duplex assay. The insertion sequence is present in up to 110 copies in the *C. burnetii* genome (Klee *et al.* 2006), and hence, this assay is likely to be more sensitive, although reports have suggested that not all strains contain this insert (Marmion *et al.* 2005 and Self *et al.* 2009). For this reason, both assays were combined in a duplex. Additionally, the use of the two targets allowed for identification of potential amplicon contamination. As the *com1* gene is present as one copy only in all known *C. burnetii* genomes (Zhang *et al.* 1997), it was used in this study to quantify the numbers of bacteria and to compare DNA extraction methods for detection of *C. burnetii* in clinical samples.

The spiking of PBS, buffy coat, plasma and serum samples with *C. burnetii* DNA prior to extraction by the silica column method showed no significant differences in the yields of bacterial DNA. A normal 4 ml blood sample would contain $1{\cdot}6 \times 10^7$–$4{\cdot}4 \times 10^7$ white blood cells. According to the manufacturer's handbook, this method can yield up to 50 μg of DNA from 200 μl of buffy coat. A greater volume of sample may increase the amount of host DNA to a level that may inhibit the qPCR detection of *C. burnetii*. This indicates the effectiveness of the silica column DNA extraction method for buffy coat, plasma and serum samples.

In Q-fever infections, *C. burnetii* may not be circulating in the blood but may be present in other tissues such as bone marrow, as demonstrated in patients with chronic Q fever (Peacock *et al.* 1983) and post-Q-fever fatigue syndrome 5 years after the primary infection (Harris *et al.* 2000) and 12 years following acute infection (Marmion *et al.* 2005). In a previous study, dilutions of the vaccine Q-Vax® (containing approximately 1×10^9 cells per 25 μg) were made in buffer. This was tested against the same preparation spiked with eukaryotic DNA from normal bone marrow samples that had been extracted by a phenol method (Harris *et al.* 2000). Samples with bone marrow DNA had a delayed C_t by approximately 12 cycles, suggesting that eukaryotic DNA from bone marrow led to a significant reduction in PCR amplification efficiency (Harris *et al.* 2000). In the current study, a comparable amount of DNA was detected in bone marrow samples compared to PBS controls spiked post-DNA extraction (data not shown). This was true for samples extracted by either the chloroform or the silica column method, suggesting that the reduction in PCR efficiency previously observed (Harris *et al.* 2000) may have been because of the suboptimal method of DNA extraction rather than the sample type.

Coxiella burnetii that is persistent in patients may be in the resistant SCV form (Marmion *et al.* 2005). Small-cell variant-enriched *C. burnetii* in cell culture were then used to determine the optimal DNA extraction methods for the SCV cell type. There was a significant ($P < 0{\cdot}05$) increase in the amount of DNA extracted by the silica column method compared with those extracted by the chloroform method. Extra extractions using numerous washes with chloroform were not necessary to lyse the SCV. A longer digestion with proteinase K improved the PCR detection of *C. burnetii* DNA slightly (although not statistically significant).

A comparison was made between DNA extraction methods for detecting SCV *C. burnetii* in spiked bone marrow samples. DNA extraction methods were compared with bone marrow samples spiked with either filtered (0·22 μm) (assumed to consist of only SCV) or unfiltered but SCV-enriched cultures. The phenol–chloroform method was the poorest among the methods tested, yielding the least amount of DNA in both the filtered and unfiltered spiked samples. The addition of a 95 °C incubation step in the chloroform method significantly decreased the amount of bacterial DNA detected. This additional step may have been expected to increase the bacterial DNA yield because of increased temperature and incubation aiding complete cell lysis. The observed decrease may have been because of inhibitors binding to the single-stranded DNA. A similar result was seen in a study showing that the inhibitory effect of IgG was increased if the sample was heated to 95 °C or if there was less nontemplate DNA present (Al-Soud *et al.* 2000). The SCV were apparently protected from these effects, as no significant difference was demonstrated with the filtered (SCV only) samples. The SCV of *C. burnetii* has condensed chromatin (McCaul and Williams 1981), which may prevent inhibitors binding and/or increase the temperature required to separate the strands of DNA, hence protecting the DNA from inhibitors binding to the separated strands. For the samples spiked with either the unfiltered or filtered cultures, the silica column method (RBC-silica) extracted the largest amounts of detectable DNA.

The comparison of different extraction methods was repeated using Q-Vax®, the human Q-fever vaccine, to spike bone marrow samples. This was based on the assumption that the formalin-killed cells in the vaccine would have less cell debris and less cell-free DNA than cell cultures. Thus, any differences in the DNA detected between the filtered and unfiltered would be because of the ability of that particular method to release DNA from intact bacterial cells and reduce the amount of PCR inhibitors. RBC lysis buffer was tested in the Q-Vax® spiked samples to determine if it had any effect on the detection of *C. burnetii* SCV. No significant differences were observed between extraction methods when used with samples spiked with unfiltered Q-Vax®. This was in contrast to the samples spiked with *C. burnetii* cell cultures, where the addition of a heating step significantly decreased the amount of DNA detected. This suggests that the formalin-killed *C. burnetii* in the Q-Vax® were more resistant to the 95 °C incubation step than the cultured cells. The samples spiked with filtered Q-Vax® demonstrated that significantly more DNA was detected in samples extracted by the silica column method than those extracted by the chloroform method. With all extraction methods, filtering of the *C. burnetii* used to spike samples reduced the amount of DNA detected. This reduction was more evident in those samples spiked with SCV-enriched *C. burnetii* cell cultures than those spiked with Q-Vax®. This may be because of the *C. burnetii* constituents of Q-Vax® which potentially had a higher concentration of SCV than the enriched cell cultures. Alternatively, Q-Vax® may have higher amounts of cell debris and hence more cell-free DNA that would pass through a 0·22-μm filter. The differences may also be because of a PCR-inhibitor residue resulting from the vaccine preparation or the phase of the *C. burnetii* cells. In cell culture, *C. burnetii* is primarily in phase II (Baca *et al.* 1981), whereas in Q-Vax®, the *C. burnetii* cells are presumed to be primarily phase I. This difference may affect the ease of opening of the *C. burnetii* cells during DNA extraction.

The results of this study complement previous studies that have compared DNA extraction methods on different sample types to optimize detection of infectious agents by PCR. Previously, it has been demonstrated that a phenol–chloroform method of extraction produces PCR inhibitors in the final eluate (Kok *et al.* 2000; Roussel *et al.* 2005; Clements *et al.* 2008). Indeed in the current study, the phenol–chloroform method (PHC) of *C. burnetii* DNA extraction was the worst method for bone marrow samples.

Both the phenol–chloroform (PHC) and the chloroform (C-Silica and 95-C-Silica) methods of DNA extraction were time-consuming, labour intensive, complicated, expensive, unsuitable for treating high numbers of samples, used hazardous chemicals and had many steps which increase the potential for technical error, loss of sample and contamination. The results of this study indicated that these methods were not necessary for the extraction of DNA from the SCV of *C. burnetii*. Indeed the optimal method for detection of *C. burnetii* was the silica column method, which was relatively simple, straightforward and is widely used in both research and diagnostic fields. Furthermore, the use of the duplex qPCR was a rapid, sensitive and specific method for detecting *C. burnetii* DNA in spiked human diagnostic samples.

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