

Interlaboratory Evaluation of Different Extraction and Real-Time PCR Methods for Detection of *Coxiella burnetii* DNA in Serum[∇]

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Received 19 May 2010/Returned for modification 15 July 2010/Accepted 27 August 2010

In the Netherlands, there is an ongoing and unparalleled outbreak of Q fever. Rapid and reliable methods to identify patients infected with *Coxiella burnetii*, the causative agent of Q fever, are urgently needed. We evaluated the performance of different DNA extraction methods and real-time PCR assays that are in use in seven diagnostic or reference laboratories in the Netherlands. A low degree of variation in the sensitivities of most of the developed real-time PCR assays was observed. However, PCR assays amplifying short DNA fragments yielded better results than those producing large DNA fragments. With regard to DNA extraction, the automated MagNA Pure Compact system and the manual QIAamp DNA mini kit consistently yielded better results than either the MagNA Pure LC system and NucliSens EasyMag (both automated) or the High Pure viral nucleic acid kit (manual). The present study shows that multiple combinations of DNA extraction kits and real-time PCR assays offer equivalent solutions to detect *C. burnetii* DNA in serum samples from patients suspected to have Q fever.

Q fever is a worldwide zoonosis caused by *Coxiella burnetii*, an obligate intracellular bacterium (11). Whereas animals such as sheep and goats are generally asymptomatic carriers, infection with *C. burnetii* in these animals may become manifest by abortion. Although asymptomatic in ~60% of infected persons, *C. burnetii* can cause serious illness in humans. Q fever can cause acute or chronic infection depending on the patient's condition or immune status. Acute Q fever may present as a self-limiting flu-like atypical pneumonia accompanied by severe headache and sometimes hepatitis. Approximately 5% of all Q fever cases may progress in a chronic infection leading to life-threatening endocarditis (1, 3, 5, 7–9). *C. burnetii* is highly infectious and can survive for long periods in the environment. Human outbreaks have been associated with farms, slaughterhouses, and wind dispersion from farms where infected animals were kept. Ticks and pets, including cats and dogs, have also been demonstrated to be potential sources of Q fever (1, 4, 10).

Laboratory diagnosis of Q fever is usually performed by serological methods such as the indirect immunofluorescence assay (IFA), complement fixation test (CFT), or enzyme-linked immunosorbent assay (ELISA), but these tests are of limited use in the early phase of the disease, as it may take

up to 2 weeks for a detectable immune response to develop. Several PCR-based diagnostic methods, such as conventional PCR, nested PCR, or real-time PCR, have successfully been applied for the direct detection of *C. burnetii* DNA in clinical samples. The sequences targeted by these tests varied from plasmids (QpH1 or QpRS) to chromosomal genes, such as the isocitrate dehydrogenase gene (NADP) or the transposase gene of the *C. burnetii* IS1111a insertion element (3, 4, 14–16). The multicopy IS1111a insertion element is present in 20 copies in the genome of the *C. burnetii* Nine Mile RSA493 strain. Copy numbers per isolate vary and can reach up to ~100 copies per genome (7). Due to the multicopy nature of this DNA element, it provides a highly sensitive target for detection of *C. burnetii* DNA in serum samples. Furthermore, real-time PCR can be useful for diagnosis of chronic Q fever, since in these patients *C. burnetii* DNA can be detected in serum over long periods of time (3).

In the Netherlands, as of 2007, there is an unprecedented and ongoing outbreak of Q fever (12, 17). At present, more than 3,000 cases have been reported in the Netherlands. In order to improve diagnosis for Q fever, medical microbiology laboratories have implemented molecular methods to close the diagnostic gap between onset of the disease and the presence of specific antibodies in serum. The aim of this study was to compare the performances of different DNA extraction methods and real-time PCR assays, all targeting the *C. burnetii* IS1111a insertion element, that are being used in seven diagnostic or public health laboratories in the Netherlands.

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[∇] Published ahead of print on 8 September 2010.

TABLE 1. Overview of equipment and protocols used by the participating laboratories

Laboratory	DNA extraction platform	DNA isolation kit	PCR platform	PCR reagents	Reaction vol (μ l)	Cycling parameters ^e
A	Manual	High Pure viral nucleic acid kit ^a	LightCycler 480 ^a	LightCycler 480 Probe Master ^a	20	95°C, 1 s; 60°C, 12 s (50 \times)
B	NucliSens EasyMag ^b	NucliSens magnetic extraction reagents ^b	ABI Prism 7500 ^c	In-house master mix	25	95°C, 15 s; 60°C, 1 min (45 \times)
C	MagNA Pure Compact system ^a	MagNA Pure Compact nucleic acid isolation kit I ^a	ABI Prism 7500	ABI Universal Master mix ^c	25	50°C, 2 min; 95°C, 15 s; 60°C, 1 min (45 \times)
D	MagNA Pure Compact system MagNA Pure LC System ^a	MagNA Pure Compact nucleic acid isolation kit I MagNA Pure LC total nucleic acid isolation kit ^a	ABI Prism 7500	ABI Universal Master mix	25	50°C, 2 min; 95°C, 15 s; 60°C, 1 min (45 \times)
E	Manual	QIAamp DNA mini kit ^d	ABI Prism 7500	ABI Universal Master mix	30	50°C, 2 min; 95°C, 15 s; 60°C, 1 min (45 \times)
F	MagNA Pure Compact system MagNA Pure LC system	MagNA Pure Compact nucleic acid isolation kit I MagNA Pure LC total nucleic acid isolation kit	LightCycler 480	LightCycler 480 Probe Master	50	95°C, 15 s; 60°C, 1 min (45 \times)
G	Manual	QIAamp DNA mini kit	LightCycler 480	LightCycler FastStart DNA Master HybProbe ^a	20	95°C, 10 s; 60°C, 20 s; 72°C, 15 s (45 \times)

^a Roche Diagnostics, Almere, Netherlands.

^b bioMerieux, Boxtel, Netherlands.

^c Applied Biosystems, Nieuwekerk a/d IJssel, Netherlands.

^d Qiagen, Venlo, Netherlands.

^e Excluding any initial denaturation or final extension and/or cooling step.

MATERIALS AND METHODS

Study design. The study was set up to separately assess the performances of the DNA extraction methods and of the real-time PCR assays. The analytical sensitivities of the different PCR methods were determined using a dilution series of genomic DNA extracted from the Nine Mile strain (RSA493). The concentration of the DNA was determined by UV measurements and adjusted to approximately 50 fg/ μ l. Three-fold serial dilutions (nine steps) were prepared in duplicate. Each DNA sample was tested in duplicate, and thus four PCR results were obtained per sample. Based on a genome size of 2 Mbp and the presence of 20 *IS1111a* copies per Nine Mile genome, the starting dilution (9-fold) contains approximately 50 target copies/ μ l DNA. The samples were blinded. All laboratories used a fixed amount of 5 μ l DNA in their PCRs according to the procedure of each individual laboratory (Table 1).

Furthermore, all laboratories received three serum samples to compare the relative efficiencies of the different DNA extraction methods. Two Q fever-positive clinical samples were actual clinical samples from patients suffering from acute Q fever. The clinical samples were qualified based on the threshold cycle (C_T) value that was obtained during initial screening of the samples. Sample A represents a strong positive sample, and sample B is on the lower end of the normal range for positive samples and repeatedly tested positive. The third sample (C) was a negative control.

All samples were provided in duplicate and blinded such that the person handling the samples had no prior knowledge of the contents. According to the protocol, each laboratory was instructed to isolate DNA from 200 μ l of each serum sample, using their routine DNA extraction method. Details of the DNA extraction procedures and amplification protocols are described in Table 1. DNA was eluted in 100 μ l of elution buffer, supplemented with 5 μ l of bovine serum albumin (New England Biolabs, Ipswich, MA) to a final concentration of 0.1 mg/ml, and stored at -20°C until further use. All DNA samples were subsequently collected by a central laboratory and redistributed among the participat-

ing laboratories for PCR analysis. Each real-time PCR mixture contained 5 μ l of DNA extract. PCR analysis of each DNA extract was performed in duplicate, and thus four PCR results were obtained per original sample. The locations of the primers and probe combinations used by the participating laboratories are shown in Fig. 1 and described in Table 2.

All samples were shipped on dry ice using an overnight delivery service. Laboratories were instructed to keep the samples frozen until they were processed.

RESULTS

In the first experiment, the analytical sensitivities of the different real-time PCR procedures were evaluated using nine samples of 3-fold serial dilutions of DNA extracted from the Nine Mile strain (RSA493). From each DNA dilution, four PCR results were obtained per laboratory. A minimum difference in PCR assay sensitivity was observed between the laboratories when evaluated on a dilution series of genomic DNA (Table 3). All laboratories were able to demonstrate the presence of *C. burnetii* DNA in four out of four replicates in the first two dilutions. Five laboratories were able to demonstrate the presence of *C. burnetii* DNA in at least two out of four replicates in the fourth dilution step. One laboratory was able to demonstrate the presence of *C. burnetii* DNA in three out of four replicates in the fifth dilution step. Incidentally, a single positive PCR result was obtained with some of the higher DNA dilutions, while a lower dilution remained PCR negative. We

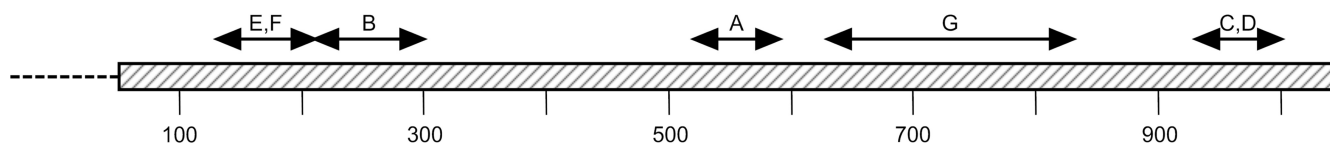


FIG. 1. Location of amplicon with PCR primers and probes from each laboratory (A to G) on the *IS1111a* insertion element.

TABLE 2. Nucleotide sequences of amplification primers and detection probes used in this study.

Laboratory(ies)	Format ^a	Primer or probe	Concn (μM)	Sequence (5' → 3')	Location ^b	Amplicon size (bp)	Reference
A	TM	QKF3	0.5	GTGGTGCCAAGCGATTTTAT	7216–7235	78	This study
		QKR3	0.5	GTTTCATCCGCGGTGTTAAT	7293–7274		
		QKP3	0.2	FAM-TTTAGCGAGCGAAGCGGTGG-TAMRA	7253–7272		
B	TM	QFw	0.9	TGATGGAAGCGTGTGGAGG	6910–6928	87	This study
		QRv	0.9	CGTGTCTGCGGACTGATCAAC	6996–6977		
		QProbe	0.2	VIC-GCGAACCATTGGTATCGGACGTTT-TAMRA	6951–6974		
C, D	TM	456	0.5	AAAACGGATAAAAAGAGTCTGTGGTT	7635–7660	70	13
		457	0.5	CCACACAAGCGCGATTCAT	7704–7686		
		Coxbur	0.15	FAM-AAAGCACTCATTGAGCGCCGCG-TAMRA	7661–7683		
E, F	TM	IS FW	0.5	AAAGTGATCTACACGAGACGGGTTA	6834–6858	75	This study
		IS REV	0.5	CACGCAGCCCACCTTAAGAC	6908–6889		
		PIS	0.2	FAM-CGTGCTCAGTATGTATC-MGB	6861–6877		
G	HP	CbISF-2	0.5	GGACGAAGCGATTGGTGATTAC	7331–7352	202	This study
		CbISR-2	0.5	ACTCGAATGTTGTCGAGGG	7532–7514		
		CbIS1111aFL	0.2	GCGTGGGTGACATTCATCAATTTTCATCG-Flu	7453–7480		
		CbIS1111aCT635	0.2	CFRed635-CCCGGCAGTTGTCTGGCGTTTA-PO ₄	7483–7503		

^a TM, TaqMan/hydrolysis probe; HP, hybridization probe.

^b Location in positions 6684 to 7778 of the whole genome sequence of *C. burnetii* RSA493 (GenBank accession number AE016828), encoding the transposase gene of the *C. burnetii*-specific *IS1111a* insertion element.

believe that this is the result of the stochastic distribution of the DNA molecules over these high dilutions.

By the design of the study, we were able to assess the relative efficiencies of the DNA extraction methods that were used as well as the sensitivities of the PCR assays being used. These were evaluated with clinical samples from Q fever patients. The results are shown in Table 4. For sample A, the percentages of positive PCRs were similar, indicating that all extraction methods performed equally well on this sample (71 to 89%). All but one of the laboratories were able to produce at least three out of four positive PCR results. With one exception, a positive PCR result was obtained in 89 to 97% of samples across all DNA extraction methods. More variation in the results was seen with sample B. Depending on the DNA extraction method used, a positive PCR result was obtained in 25 to 64% across all real-time PCR assays. A positive PCR result was obtained in 3 to 78% of samples across all DNA extraction methods. Apparently, the sensitivity of one of the PCR assays was much lower for both samples on DNA extracted from clinical samples than those of the other assays

(laboratory G). The PCR assay that was used by laboratories C and D showed the highest overall sensitivity on DNA extracted from serum. With one exception (laboratory G), none of the laboratories obtained a positive PCR result on the negative-control sample (sample C) (data not shown).

All PCR primers and probes were designed using various primer and probe design programs targeting the DNA sequence of the *IS1111a* insertion element from the genome of the Nine Mile strain RSA493 (GenBank accession number AE016828). More recently, the genomic sequences of four additional *C. burnetii* strains, i.e., Dugway (accession number CP000733), RSA331 (CP000890), CbuG Q212 (CP001019), and Cbuk Q154 (CP001020), have become available from the National Center for Biotechnology Information (NCBI) nucleotide database (<http://www.ncbi.nlm.nih.gov>). The insertion elements of these strains may contain polymorphisms that have not been taken into account in development of the PCR primers and probes. Since any mismatch between the primer or probe sequence and the target sequence may lead to less efficient or less sensitive PCR assays, the suitability of the developed primers and probes was reassessed *in silico* using these additionally available genomic sequences. As it turns out, certain primer and probe combinations contain mismatches in the target sequences that may affect the performance of the PCR assays (Table 5).

TABLE 3. Number of positive PCRs using a 3-fold serial dilution series of Nine Mile DNA tested with four replicates

Dilution (fold)	Approximate copy no. per DNA sample (5 μl)	No. of positive PCRs in laboratory:						
		A	B	C	D	E	F	G
9	250	4	4	4	4	4	4	4
27	83	4	4	4	4	4	4	4
81	28	4	4	2	4	3	4	1
243	9.3	2	1	3	2	2	2	1
729	3.1	3	1	0	0	0	1	0
2,187	1.0	1	0	1	0	0	1	1
6,561	0.34	0	1	0	0	1	0	0
19,683	0.11	0	0	0	0	0	0	0
59,049	0.04	0	0	0	0	0	0	0

DISCUSSION

Reliable detection of *C. burnetii* DNA in serum requires highly efficient DNA extraction procedures and sensitive PCR methods. In this study we compared the analytical procedures that were developed by diagnostic laboratories in the Netherlands to aid in identification of patients with Q fever. All PCR assays targeted the *C. burnetii* *IS1111a* element, a multicopy element that has been reported to be specific for *Coxiella burnetii* (2, 7, 16). By the design of this study, we were able to

TABLE 4. Efficiency of DNA extraction methods and sensitivity of real-time PCR assays (tested in quadruplicate)

Sample	DNA extraction method (laboratory)	No. of positive results out of 4 PCRs in laboratory:							Total % positive
		A	B	C	D	E	F	G	
A ^a	High Pure viral nucleic acid kit (A)	3	4	3	4	3	3	0	71
	QIAamp DNA mini kit (E)	4	4	4	4	4	4	1	89
	QIAamp DNA mini kit (G)	4	4	4	4	4	4	0	86
	NucliSens EasyMag (B)	3	3	4	3	4	3	0	71
	MagNA Pure Compact (C)	4	4	4	4	4	4	1	89
	MagNA Pure Compact (D)	4	3	4	4	4	4	0	82
	MagNA Pure Compact (F)	3	4	4	4	4	4	0	82
	MagNA Pure LC system (D)	4	3	4	4	3	3	0	75
	MagNA Pure LC system (F)	4	3	4	4	4	4	0	82
Total % positive	92	89	97	97	94	92	6		
B ^b	High Pure viral nucleic acid kit (A)	2	1	2	3	1	0	0	32
	QIAamp DNA mini kit (E)	2	1	3	4	3	4	0	61
	QIAamp DNA mini kit (G)	1	2	3	4	4	2	0	57
	NucliSens EasyMag (B)	1	1	2	0	2	1	0	25
	MagNA Pure Compact (C)	3	1	4	4	2	4	0	64
	MagNA Pure Compact (D)	1	0	4	3	2	3	1	50
	MagNA Pure Compact (F)	2	1	2	4	4	3	0	57
	MagNA Pure LC system (D)	0	1	1	2	4	1	0	32
	MagNA Pure LC system (F)	1	0	2	4	0	2	0	32
Total % positive	36	22	64	78	61	56	3		

^a Clinical positive sample with a relatively high DNA load.

^b Clinical positive sample with a relatively low DNA load.

separately analyze the performance of the DNA extraction procedure and of the PCR assays. In this study, we used actual clinical serum samples containing different loads of *C. burnetii* (DNA) instead of serum samples that were spiked with high-molecular-weight genomic DNA from *C. burnetii* DNA to get a more representative measure of the efficiency of the DNA extraction methods used. Manual extraction procedures performed equally as well as some automated extraction platforms. Despite the fact that most commercial DNA extraction

kits are based on the similar chemistries, the automated MagNA Pure Compact system and the manual QIAamp DNA mini kit consistently yielded better results than either the MagNA Pure LC system and the NucliSens EasyMag (both automated) or the High Pure viral nucleic acid kit (manual). Less variation was observed in the sensitivity of the different PCR assays, with one major exception: the PCR that was in use in laboratory G was much less sensitive than the other PCR assays. This PCR performed similarly to the other assays on a dilution series of high-molecular-weight genomic DNA from the Nine Mile strain but was less sensitive on DNA extracted from clinical samples. Unfortunately, insufficient material was left to confirm these findings. However, there appears to be a very plausible explanation for these results: the lack in sensitivity may possibly be explained by the significantly larger PCR product that is targeted by laboratory G (202 bp, versus 70 to 87 bp in the other laboratories). In general cell-free DNA in serum is very likely to be degraded to a certain extent. Therefore, short sequences are likely to represent better targets than larger sequences. The highest overall sensitivity on DNA extracted from serum was obtained by the PCR assay that was in use in laboratories C and D. Notably, although the differences from most other PCR assays were relatively small, this assay targeted the shortest DNA fragment (70 bp). Another explanation for this difference may reside in sequence polymorphisms in the target sequences. All PCR assays were developed using the *IS1111a* sequence from the Nine Mile RSA493 strain. Analysis of *IS1111a* sequences from various other *C. burnetii* genomes shows the presence of multiple nucleotide polymorphisms that may affect PCR performance. Certain PCR amplification primers or detection probes do not match the target sequences of certain genomes equally well (Table 5). Depending on the location of the present mismatch(es), this may have had a detrimental effect on the performance of the

TABLE 5. Numbers of *IS1111a* insertion elements with exact matches to the primer and probe sequences in the genomes of five different *C. burnetii* isolates

Laboratory(ies)	No. of elements in strain (<i>IS1111a</i> copy no.):				
	RSA493 (20)	Dugway (12)	RSA331 (47)	CbuG Q212 (28)	CbuK Q154 (48)
A	20	12	43 ^b	28	48
B	20	12	47	28	48
C, D	20	12	47	28	40 ^e
E, F	20	0 ^a	47	28	46 ^f
G	20	12	46 ^c	0 ^d	8 ^g

^a This involves a single nucleotide mismatch at the 5' end of the forward primer.

^b This involves various mismatches in both forward and reverse primers in 4 of the 47 copies of the *IS1111a* insertion element.

^c This involves six mismatches in the reverse primer in 1 of the 47 copies of the *IS1111a* insertion element.

^d This involves a single nucleotide mismatch at a central position of the 28-base CbIS1111aFL probe.

^e This involves a single nucleotide mismatch at the second position of the forward primer in 8 of the 48 copies of the *IS1111a* insertion element.

^f This involves a single nucleotide mismatch and a double nucleotide mismatch in the reverse primer, both in 1 of the 48 copies of the *IS1111a* insertion element.

^g This involves a single nucleotide mismatch at the 5' end of the reverse primer in 29 of the 48 copies of the *IS1111a* insertion element and various mismatches in the reverse primers in another 10 of the 48 copies of the *IS1111a* insertion element.

PCR, especially for those near the 3' end of the primers or near the 5' end of a hydrolysis probe. It has been shown that *C. burnetii* genotypes from the Netherlands display a considerable degree of homogeneity, indicating a clonal origin (6). Therefore, DNA sequence polymorphisms in the IS1111a elements in this Dutch clone may also affect the different PCR assays studied here and may also explain the poor performance of the PCR used by laboratory G. Whether or not this is a likely explanation remains to be established. Obviously, in samples with a very low target concentration, sampling variation may explain part of the heterogeneity seen in the results from the dilution series of genomic DNA from the Nine Mile strain (Table 3). For the evaluation of the performance of the DNA extraction methods, all participating laboratories analyzed all of the extracted DNA samples, and any effect of sampling variation on the interpretation of the results would be minimized. Two different real-time PCR platforms were used by the participating laboratories (LightCycler 480 or ABI Prism 7500). Although this could have contributed to differences in sensitivities for the various assays, in the context of the results presented in Table 3 and the alternative explanations for the lower sensitivity of the assay used by laboratory G, this appears unlikely to be a contributing factor.

In conclusion, we show that multiple combinations of DNA extraction kits and real-time PCR assays offer equivalent solutions for the screening of patients suspected to have Q fever.

ACKNOWLEDGMENTS

We thank Elise Kraan and Linda Poort for technical assistance.

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