



National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

**Detection of *Coxiella burnetii* by
(q)PCR: a comparison of available
assays**

RIVM Letter report 330071002/2011
A. de Bruin



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Abstract

Detection of *Coxiella burnetii* using (q)PCR: a comparison of available assays

Q fever, caused by the bacterium *Coxiella burnetii*, has become an emerging public health problem in the Netherlands since 2007. Diagnosis of Q fever, both in humans and animals, is mainly based on serology. Serological techniques are less suitable for direct transmission and source-finding studies for *C. burnetii* infection due to the delayed detection window for serological tests.

The last two decades, several PCR based diagnostic assays (conventional PCR or qPCR) have been developed for the detection of *C. burnetii* DNA. These assays have been applied for the detection of *C. burnetii* DNA in clinical samples, veterinary samples, and environmental samples. These PCR-based diagnostic tests are often "in-house" developed assays. A number of commercial PCR diagnostic tests, however, have also become available. A drawback of these commercial kits is that information on some of the components is patented. This makes a thorough assessment of assay performance in relation to other "in-house" developed assays difficult.

In the current study, we describe a comparison of various (q)PCR assays used by laboratories both nationally and internationally for the detection of *C. burnetii* DNA. We compare the results obtained from three ring trials, set up specifically for the detection of *C. burnetii* DNA in human, veterinary, or environmental matrices. In addition, we compare specific parameters, such as sensitivity, specificity, and reproducibility for each of the (q)PCR assays.

In conclusion, most (q)PCR assays developed for *C. burnetii* include detection of the multicopy insertion element *IS1111*, in combination with detection of other single copy genes such as *icd*, *com1*, *sod*, or plasmid genes. PCR based detection assays (conventional PCR or qPCR) for *C. burnetii* DNA preferably target short and multiple target sequences, including an internal process control in multiplex format. Other performance characteristics have not yet been published for these qPCR assays for the detection of *C. burnetii*, although this would be recommended.

Keywords:

Coxiella burnetii, PCR, Detection, Q fever

Trefwoorden:

Coxiella burnetii, PCR, Detectie, Q-koorts

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1 Available (q)PCR assays for detection of *C. burnetii* DNA

Q fever is a zoonosis caused by *Coxiella burnetii*, a gram negative, highly pleomorphic, obligate, intracellular bacterium that affects both humans and animals worldwide (15, 19). In the Netherlands, unprecedented Q fever outbreaks were reported between 2007 and 2010 (11, 20, 23, 28).

Diagnosis of Q fever, both in humans and animals, is mainly based on serology. Drawbacks of serological techniques include a delay of diagnosis because *C. burnetii* specific antibodies appear several weeks after infection and the detection of these antibodies sometimes even months after the initial infection. For these reasons serology is less suitable for direct transmission and source-finding studies for *C. burnetii* infection than is (q)PCR.

Several PCR-based diagnostic assays have been developed for *C. burnetii* and have been used for detection of *C. burnetii* DNA in clinical samples (1, 2, 4, 17, 32, 33), veterinary samples (9, 14, 18, 29), and environmental samples (12, 22, 25, 31). PCR-based methods target one or more specific sequences in the genome, most often in separate (singleplex) assays. Signature sequences most commonly used for the detection of *C. burnetii* DNA are plasmid sequences (QpH1 or QpRS), or chromosomal genes such as isocitrate-dehydrogenase (*icd*), the outer membrane protein coding gene *com1*, the superoxide dismutase gene (*sod*), or the transposase gene in insertion element *IS1111*. The latter target is a preferred target for PCR assays due to its presence in multiple copies within the genome, thereby enhancing sensitivity of detection (13, 26). However, since the number of *IS1111* copies in the *C. burnetii* genome varies between strains, direct quantification of the number of organisms within a sample using this target is not straightforward (14).

A large number of the PCR based diagnostic tests described in the literature are "in-house" developed assays, designed for the detection of *C. burnetii* DNA in specific human, veterinary, or environmental matrices. However, a number of commercial PCR diagnostic tests is also available. For instance, the LSI – kit TaqVet™ *Coxiella burnetii* (LSI - Laboratoire Service International, France), the LightMix® (TIB MOLBIOL GmbH, Germany), or AdiaVet Cox (Adiagene, France). A drawback of commercial kits is that information on some of the components (PCR Mastermix reagents, primer & probe sequences, PCR product lengths) is patented. This makes a thorough assessment of assay performance in relation to other published "in-house" developed assays difficult.

In the current study, we describe a comparison of various (q)PCR assays used by laboratories both nationally and internationally for the detection of *C. burnetii* DNA. We compare the results obtained from three ring trials, specifically set up for the detection of *C. burnetii* DNA in human, veterinary, or environmental matrices. In addition, we compare specific parameters, such as sensitivity, specificity, efficiency, and reproducibility of each (q)PCR assay (when available), as described in "The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments" (5).

2 International and national ring trials for the detection of *C. burnetii* DNA using (q)PCR

To evaluate the different (q)PCR assays used for the detection of *C. burnetii*, a number of national and international ring trials were initiated between 2008 and 2010. Currently, results of three separate ring trials are published (3, 10, 27).

The first ring trial (3) was initiated by the National Institute for Public Health and the Environment in the Netherlands (RIVM). This ring trial was set up to compare (q)PCR assay results obtained from DNA extracts of PCR inhibiting environmental matrices. The second ring trial (10) was initiated by the Veterinary Laboratories Agency in the United Kingdom (VLA) and was also a comparison of different (q)PCR assays only. This ring trial focussed primarily on testing DNA extracts obtained from veterinary sources.

The third ring trial (27) was initiated by the Canisius Wilhelmina Hospital in the Netherlands (CWZ). This ring trial was set up to compare both DNA extraction procedures and (q)PCR assay results. The main focus of this ring trial was on human serum samples.

A summary of the set up and outcome of all three ring trials is described in the next paragraphs.

2.1 Ring trial for detection of *C. burnetii* DNA in environmental samples

Within the EmZoo project, a research project on emerging zoonoses and coordinated by RIVM (3), a ring trial was organised for the detection of *C. burnetii* DNA in environmental samples. In this ring trial, organised by the laboratory for Zoonoses and Environmental Microbiology (RIVM-LZO), seven national laboratories participated. The laboratory for Infectious diseases and perinatal screening (RIVM-LIS) and RIVM-LZO were representatives of the National Institute for Public Health and the Environment (RIVM). The Central Veterinary Institute (CVI) and the Animal Health Service (GD) were representatives of the animal health agencies. In addition, TNO-Defence Security and Safety (TNO D&V), The Jeroen Bosch Hospital in 's Hertogenbosch and the Pathology and Medical Microbiology Foundation (Stichting PAMM) in Veldhoven also participated in this comparison of (q)PCR assays for *C. burnetii*.

Each testing laboratory received a panel of fifteen blinded nucleic acid samples coded A-O. Samples A, D, G, and J were dilutions prepared from a gDNA stock of the *C. burnetii* nine-mile strain (RSA 493). Samples C, F, I, L, and O were complex, PCR inhibiting DNA extracts obtained from an environmental matrix (dust), and samples B, E, H, K, and N were *C. burnetii* DNA samples prepared by the Central Veterinary Institute (CVI). Sample M was a negative control sample, containing only H₂O. Each participating laboratory tested the panel of nucleic acid extracts using their 'in house' real-time (q)PCR assay(s). The amount of DNA template to be added to the PCR mixtures was restricted to 3 µl per reaction.

The most common target selected for real-time PCR assays for *C. burnetii* by the participating laboratories is the *IS1111* insertion element that is present in multiple copies in the *C. burnetii* genome. Some laboratories use additional real-time PCR assays for *C. burnetii* detection (often in multiplex format), in which single copy genes like *com1* or *sod*, were also targeted. The results for each participating laboratory are summarized in Table 1. Results are indicated with the symbols + (Positive, or *C. burnetii* detected), +D (Positive at 10x dilution),

+* (*C. burnetii* detected nearby detection limit, or dubious results), - (Negative, or no *C. burnetii* detected), and ? (positive water control: not determined). Information regarding real time PCR platforms and PCR reaction conditions are also given in Table 1.

Based on the number of samples detected, the RIVM-LIS qPCR assay scored the best results regarding positive detection of undiluted samples (12 out of 15). The qPCR assays of RIVM-LZO and CVI (12/15, with three samples scoring positive after 10x dilution), JBZ (9/15), PAMM (8/15), GD (7/15) and TNO D&V (5/15) followed. A more extensive description of the ring trial was published in the EMZOO report entitled "Emerging Zoonoses: Early warning and surveillance in the Netherlands" (3).

2.2 Ring trial for detection of *C. burnetii* DNA in veterinary samples

Seven international laboratories participated in a ring trial for the detection of *C. burnetii* DNA, initiated by the Veterinary Laboratories Agency (VLA) in the United Kingdom. The results of this ring trial, which tested various qPCR assays on DNA extracts obtained from veterinary sources, were published in 2011 (10). The participating laboratories were: the Veterinary Laboratories Agency (United Kingdom), the Federal Institute for Risk Assessment (Germany), the Health Protection Agency (United Kingdom), the National Veterinary Institute (Sweden), Royal Hospitals, Belfast HSC Trust (United Kingdom), National Institute for Public Health and the Environment (RIVM-LZO, the Netherlands), and the Central Veterinary Institute (the Netherlands).

Each testing laboratory received a panel of ten blinded nucleic acid samples, obtained from placental material or fetal fluids of Caprine, Ovine, or Bovine origin. Each participating laboratory tested the panel of nucleic acid extracts using their "in house" real-time PCR assay(s), a commercial kit (Adiavet Cox, France), or (q)PCR assays already published (8, 14, 16). The amount of DNA template to be added to the PCR mixtures was not restricted and no ranking was applied to the performance of the different (q)PCR assays.

It was shown that when the Cq values for each positive sample were examined, there is close agreement between the results obtained with the real-time PCR methods that target the same region of the *C. burnetii* genome. In addition, instead of ranking particular (q)PCR assays, ranking was applied to the different target sequences within (q)PCR assays used for the detection of *C. burnetii* DNA. As expected, the multicopy insertion element *IS1111* showed the highest detection capability of all target sequences, followed by single copy target sequences such as: *com1*, *icd*, *16S*, and a hypothetical protein target sequence *HYP0*. Another conclusion of the ring trial was that, although, insertion element *IS1111* is a highly sensitive target sequence for the detection of *C. burnetii* DNA, it cannot be used for quantification purposes due to variation in the number of copies within and between the different *C. burnetii* strains.

2.3 Ring trial for detection of *C. burnetii* DNA in clinical samples

Seven national laboratories participated in a ring trial for the detection of *C. burnetii* DNA in serum, initiated by the Canisius Wilhelmina Hospital in the Netherlands. The results of this ring trial, testing both DNA extraction procedures and qPCR concomitantly were also published (27).

The participating laboratories were: the Canisius Wilhelmina Hospital (Nijmegen), Radboud University Medical Center (Nijmegen), VU University Medical Center (Amsterdam), St. Elisabeth Hospital (Tilburg), Jeroen Bosch

Hospital ('s-Hertogenbosch), St. Antonius Hospital (Nieuwegein), and RIVM-LIS of the National Institute for Public Health and the Environment (Bilthoven).

The study was set up to separate the assessment of DNA extraction procedures from the real-time (q)PCR assays. 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 third sample was a negative control.

In addition to the DNA extracts obtained from the serum samples, each participating laboratory tested a panel of a dilution series of genomic DNA, extracted from the Nine Mile strain (RSA493). All samples were tested using "in house" real-time PCR assay(s), or (q)PCR assays already published (24).

All (q)PCR assays tested targeted the DNA sequence of the *IS1111* insertion element only. The amount of DNA template to be added to the PCR mixtures was set at 5 µl per reaction.

The study showed a low degree of variation in the sensitivities of most of the real-time (q)PCR assays. In the comparison between DNA extraction procedures, 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 highest overall sensitivity on DNA extracted from serum was obtained by the qPCR assay that was in use in laboratories C and D. This may be explained by the finding that (q)PCR assays amplifying relatively short DNA fragments, as in the tests by C and D yielded better results than assays targeted at longer DNA fragments.

Table 1. Results of the ring trial initiated by RIVM for the comparison of (q)PCR assays in the detection of *C. burnetii* DNA.

Sample	Sample description	RIVM-LIS		RIVM-LZO	CVI	JBZ	TNO D&V			GD R&D	GD Routine	PAMM
		<i>IS1111</i> & <i>com1</i>	<i>IS1111</i>	<i>IS1111</i> & <i>com1</i>	<i>IS1111</i> & <i>hypo*</i>	<i>IS1111</i>	<i>com1</i>	<i>QpH11f1f2</i>	<i>sod</i>	<i>IS1111</i>	<i>IS1111</i>	<i>IS1111</i>
A	gDNA <i>C.burnetii</i> 10 ⁻⁴	+	+	+	+	+	+	-	+	+	+	+
D	gDNA <i>C.burnetii</i> 10 ⁻⁵	+	+	+	+	+	-	-	-	+	+	+
G	gDNA <i>C.burnetii</i> 10 ⁻⁶	+	+	+	+	+	-	-	-	+	+	+
J	gDNA <i>C.burnetii</i> 10 ⁻⁷	+	-	+	+	+	?	-	-	-	-	+
M	N.C. (Milliq)	-	-	-	-	-	?	-	-	-	-	-
B	CVI <i>C. burnetii</i> DNA sample nr.2	+	+	+	+	+	+	-	-	+	+	+
E	CVI <i>C. burnetii</i> DNA sample nr.4	+	+	+	+	+	-	-	-	+	+	+
H	CVI <i>C. burnetii</i> DNA sample nr.6	+	-	+	+	+	-	-	-	-	-	+
K	CVI <i>C. burnetii</i> DNA sample nr.8	-	-	-	-	-	?	-	-	-	-	-
N	CVI <i>C. burnetii</i> DNA sample nr.10	-	-	-	-	-	?	-	-	-	-	-
C	gDNA <i>C.burnetii</i> 10 ⁻¹ in PCR inhibiting background	+	+	+	+	+	+	+	+	+	+	+
F	gDNA <i>C.burnetii</i> 10 ⁻² in PCR inhibiting background	+	+	+	+	+	+	+	+	+	+	-
I	gDNA <i>C.burnetii</i> 10 ⁻³ in PCR inhibiting background	+	+	+ D	+ D	-	?	+	+	-	-	-
L	gDNA <i>C.burnetii</i> 10 ⁻⁴ in PCR inhibiting background	+	+	+ D	+ D	-	?	-	+	-	-	-
O	gDNA <i>C.burnetii</i> 10 ⁻⁵ in PCR inhibiting background	+	+	+ D	+ D	-	?	-	-	-	-	-
	Real time PCR Platform	Roche LightCycler 480	Roche LightCycler 480	Roche LightCycler 480	Applied 7500 Fast Real Time PCR system	ABI Prism 7500 SDS apparatus	Roche LightCycler 2.0			Applied Biosystems 7500 Fast Real-Time PCR System		Not supplied
	Multiplex or Singleplex reaction	Multiplex	Singleplex	Multiplex	Multiplex	Singleplex	Singleplex	Singleplex	Singleplex	Singleplex	Singleplex	Singleplex
	Reaction mix manufacturer	Roche	Roche	BioRad	Quanta	in-house'	Roche	Roche	Roche	LSI	LSI	AB
	Reactie volume (µl)	25	20	20	20	25	20	20	20	unknown	unknown	20
	DNA template added (µl)	3	3	3	5	3	3	3	3	3 or 5	3 or 5	3
	DNA enzyme activation	10 min / 95°C	10 min / 95°C	5 min / 95 °C	1 min / 95 °C	30 s / 95 °C	5 min / 95 °C	5 min / 95 °C	5 min / 95 °C	10 min / 95 °C	10 min / 95 °C	20 s / 95 °C
	Amplification & detection	10 s / 95°C	10 s / 95°C	5 s / 95 °C	10 s / 95 °C	3 s / 94 °C	10 s / 95 °C	10 s / 95 °C	5 s / 55 °C	15 s / 95 °C	15 s / 95 °C	3 s / 95 °C
		30 s / 60°C	20 s / 60°C, 15 s / 72°C	35 s / 60 °C	30 s / 60 °C	30 s / 60 °C	45 s / 60 °C	45 s / 60 °C	5 s / 55 °C, 1 min 72 °C	1 min / 60 °C	1 min / 60 °C	30 s / 60 °C
	PCR cycles	45	45	40	50	45	40	40	40	40	40	50

+	Positive, or <i>C. burnetii</i> detected
+ D	Positive, or <i>C. burnetii</i> detected at 10x dilution
+*	<i>C. burnetii</i> detected nearby detection limit, or dubious results
?	Positive water control: not determined
-	Negative, or no <i>C. burnetii</i> detected

3 Evaluation of performance characteristics of different *C. burnetii* (q)PCR assays

The comparisons made in the three published ring trials for the detection of *C. burnetii* DNA show that insertion element *IS1111* is the most used target sequence in (q)PCR based detection assays for *C. burnetii*. Many laboratories add single copy genes (chromosomal sequences like *icd*, *com1*, *sod*, or plasmid sequences like QpH1 and QpRS) to their detection assays as extra controls, or for quantification purposes.

An assessment of the performance of the various (q)PCR assays remains difficult. The number of samples, in which *C. burnetii* DNA was detected, is often the criterion on which the comparisons are based. The platforms and reagents used for (q)PCR assays were sometimes compared also. However, other important performance characteristics such as sensitivity, specificity, efficiency and reproducibility were not taken into account and are very important parameters when scoring the performance of (q)PCR assays.

3.1 Assessment of (q)PCR performance for detection of *C. burnetii* DNA

In 2009, guidelines were published for the minimum information needed for publication of the results of quantitative real-time PCR (qPCR) experiments (5). These guidelines were initiated, motivated by a lack of consensus on the assessment of qPCR assay performance and interpretation in the literature. This is illustrated by a lack of sufficient experimental detail in many publications, which impedes a reader's ability to evaluate critically the quality of the results presented or to repeat the experiments. This reduces experimental transparency and makes it difficult to compare results between laboratories. The set of guidelines describe the minimum information necessary for evaluating qPCR experiments. To evaluate qPCR performance, information is required on (a) analytical sensitivity, (b) analytical specificity, (c) accuracy, (d) repeatability, and (e) reproducibility of the assay. In the next paragraph these guidelines are summarised.

Analytical sensitivity refers to the minimum number of copies in a sample that can be measured accurately with an assay. Typically, sensitivity is expressed as the limit of detection (LOD), which is the concentration that can be detected with reasonable certainty (95% probability is commonly used) with a given analytical procedure. The most sensitive LOD theoretically possible is 3 copies per PCR (30), assuming a Poisson distribution, a 95% chance of including at least 1 copy in the (q)PCR, and single copy detection. Experimental results less than the theoretically possible LOD should never be reported.

Analytical specificity refers to the qPCR assay detecting the appropriate target sequence rather than other, non-specific targets also present in a sample. This can be assessed by testing a large panel of (closely related) non-target organisms to investigate any cross-reactivity with other species. In addition, strain coverage, and strain specificity, can be assessed by testing a large panel of different strains of the organisms.

Accuracy refers to the difference between experimentally measured and (theoretical) actual concentrations, presented as fold changes or copy number estimates.

Repeatability (short-term precision or intra-assay variance) refers to the precision and robustness of the assay with the same samples repeatedly analyzed in the same assay. It may be expressed as the standard deviation (SD) for the C_q (quantification cycle) variance.

Reproducibility (long-term precision or inter-assay variance) refers to the variation in results between runs or between different laboratories and is typically expressed as the SD or Coefficient of variance (CV) of copy numbers or concentrations.

To study all these parameters in detail in ring trials is probably not feasible, because of the costs involved. What can be concluded from the data obtained in the ring trials for *C. burnetii* detection is that a reliable PCR-based method for detection and quantification of *C. burnetii* DNA should include at least one single copy marker for quantification and a multi-copy target (e.g. *IS1111*) for enhancing sensitivity. The assay should be designed preferentially in multiplex format including an internal process control to assess PCR amplification. Multiplexing PCR detection offers several advantages, including reduction of sample volume and handling time (reducing the analysis time, cost and opportunities for lab contamination). Also, false-negative results can be reduced through co-amplification of internal controls in each sample. Using multiple redundant genetic markers for each organism reduces the chance that strain variants are missed. For instance, there has been a debate on the existence of *C. burnetii* strains missing the *IS1111* repetitive element (16, 21). Amplification of multiple signature sequences per organism will also reduce false-positive results in complex samples. False positives can be an issue if detection relies on single targets due to the presence of homologous sequences in related organisms, or unknown sources when analyzing environmental samples.

A general conclusion on “the best” (q)PCR assay for the detection of *C. burnetii* DNA cannot be derived, based on the published results in the ring trials.

For a more detailed assessment of (q)PCR performance, parameters such as sensitivity, specificity, accuracy, repeatability, and reproducibility should be taken into account. However, as pointed out by Bustin *et al.* 2010, these parameters are rarely investigated or published. For some of the (q)PCR assays used in the ring trials (and other studies), a summary of these parameters (when available), is presented in Table 2.

Table 2. Comparison a various (q)PCR assay performance parameters for a number of commonly used detection assays for *C. burnetii* DNA.

Reference	Target sequences	Amplicon size (bp)	Used in ring trial	Sensitivity ^a	Specificity tested ^b	Reproducibility ^c	Efficiency (%)
De Bruin <i>et al.</i> 2011 (6)	<i>icd</i>	139	Yes	13.0	Yes (30)	0.1	98.9 ± 0.8
	<i>com1</i>	133	Yes	10.6	Yes (30)	0.2	97.2 ± 1.5
	<i>IS1111</i>	146	Yes	10.4	Yes (30)	0.1	98.2 ± 0.9
	<i>cry1</i>	132	Yes	n.d.	Yes (30)	0.2	98.7 ± 0.7
De Bruin <i>et al.</i> 2011 (7) (submitted)	<i>com1</i>	74	Yes	10.9	Yes (30)	0.1	98.3 ± 1.0
	<i>IS1111</i>	75	Yes	8.8	Yes (30)	0.1	98.9 ± 0.6
	<i>cry1</i>	84	Yes	n.d.	Yes (30)	0.2	99.4 ± 0.5
Klee <i>et al.</i> 2006 (14)	<i>icd</i>	76	Yes	10	Yes (29)	0.2-0.6	± 90
	<i>IS1111</i>	295	Yes	6.5	Yes (29)	0.2-0.5	± 90
Schneeberger <i>et al.</i> 2010 (24)	<i>IS1111</i>	70	Yes	4	Yes (9)	1.2	99.9
Howe <i>et al.</i> 2009 (9)	<i>com1</i>	n.d.	No	24.4–36.6*	Yes (136)	n.d.	n.d.
	<i>IS1111</i>	n.d.	No	4.9*	Yes (136)	n.d.	95
Panning <i>et al.</i> 2008 (18)	<i>IS1111</i>	86	No	14-21	Yes (24)	n.d.	98

*Genome equivalents

n.d. = not determined

^aValues displayed represent the lowest DNA concentration at which 95% of the positive samples are detected

^bBetween brackets are the number of organisms tested in the non-target panel.

^cValues represent the average from the standard deviations calculated at various replicate Cq measurements.

4 Conclusions


- Insertion element *IS1111* is the most common used target sequence in (q)PCR assays designed for detection of *C. burnetii* DNA. However, this target sequence only cannot be used for quantification of *C. burnetii* DNA.
- Single copy genes (such as *icd*, *com1*, or *sod*) are often included in (q)PCR assays designed for the detection of *C. burnetii* DNA, for accurate quantification and the prevention of false positive results.
- Internal process controls should be included in (q)PCR assays to control both DNA extraction efficiency and (q)PCR amplification.
- A general conclusion on “the best” (q)PCR assay for the detection of *C. burnetii* DNA cannot be derived, based on the published results in the ring trials.
- For a thorough examination of (q)PCR performance, parameters such as sensitivity, specificity, efficiency, reproducibility, and repeatability should be reported.

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