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Detection of *Coxiella burnetii* in Complex Matrices by Using Multiplex Quantitative PCR during a Major Q Fever Outbreak in The Netherlands[▽]

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Q fever, caused by *Coxiella burnetii*, is a zoonosis with a worldwide distribution. A large rural area in the southeast of the Netherlands was heavily affected by Q fever between 2007 and 2009. This initiated the development of a robust and internally controlled multiplex quantitative PCR (qPCR) assay for the detection of *C. burnetii* DNA in veterinary and environmental matrices on suspected Q fever-affected farms. The qPCR detects three *C. burnetii* targets (*icd*, *com1*, and IS*1111*) and one *Bacillus thuringiensis* internal control target (*cry1b*). *Bacillus thuringiensis* spores were added to samples to control both DNA extraction and PCR amplification. The performance of the qPCR assay was investigated and showed a high efficiency; a limit of detection of 13.0, 10.6, and 10.4 copies per reaction for the targets *icd*, *com1*, and IS*1111*, respectively; and no cross-reactivity with the nontarget organisms tested. Screening for *C. burnetii* DNA on 29 suspected Q fever-affected farms during the Q fever epidemic in 2008 showed that swabs from dust-accumulating surfaces contained higher levels of *C. burnetii* DNA than vaginal swabs from goats or sheep. PCR inhibition by coextracted substances was observed in some environmental samples, and 10- or 100-fold dilutions of samples were sufficient to obtain interpretable signals for both the *C. burnetii* targets and the internal control. The inclusion of an internal control target and three *C. burnetii* targets in one multiplex qPCR assay showed that complex veterinary and environmental matrices can be screened reliably for the presence of *C. burnetii* DNA during an outbreak.

Q fever is a zoonosis caused by *Coxiella burnetii*, a Gram-negative, intracellular bacterium that affects both humans and animals worldwide (21, 25, 27). *Coxiella burnetii* resides and multiplies in phagolysosomes and can survive in infected cells without affecting viability (29). Due to its long persistence in the environment (11, 33) and its low infectious dose (5), it has been classified as a category B bioterrorism agent by the Centers for Disease Control and Prevention (CDC) in the United States.

Infected animals, like goats, sheep, and cattle, often show no clinical signs of infection except for abortions or stillbirths that may occur due to the infection of the placenta (2, 21, 25).

In humans, *C. burnetii* may cause serious illness, and infection is thought to occur predominantly via aerosols generated by infected animals or animal products (4, 21).

An acute Q fever infection in humans often is characterized by high fevers, headaches, and an atypical pneumonia. Endocarditis and/or hepatitis are observed most often when the disease has become chronic, which requires long-lasting antibiotic treatment (21, 25).

During the springs and summers of 2007, 2008, and 2009, large Q fever outbreaks occurred in the Netherlands affecting mainly a rural area in the southeast of the country. Prior to and during these outbreaks, abortion waves were reported on several dairy goat farms in the same region (42).

In the affected region an epidemiological study was performed in 2007, and although one goat farm with an unusually high level of abortions was thought to be involved, the exact location of the source of contamination could not be established (15). After this first outbreak, a much larger outbreak occurred in the same period in 2008, affecting the same rural area as that in 2007 and spreading over a larger geographic region (18, 34).

The diagnosis of Q fever, both in humans and animals, is based mainly on serology. Serological methods used for the detection of *C. burnetii* are indirect immunofluorescence, complement fixation, or enzyme-linked immunosorbent assays (32, 36). A drawback of these techniques is that diagnosis is delayed because *C. burnetii*-specific antibodies appear several weeks after infection and can be detected months after an infection. Serology is, for these reasons, less suitable for direct transmission and source-finding studies for *C. burnetii* infection; for that purpose PCR often is more suitable.

Several PCR-based diagnostic assays have been developed for the detection of *C. burnetii* DNA and have been used primarily for clinical samples (22, 38, 43). More recently, other types of PCR assays, like nested PCR (41, 44) and quantitative PCR (qPCR) (6, 7, 16, 17), have been developed, sometimes in combination with high-throughput capabilities (26). PCR-

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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 IS*1111*. The latter target is a preferred target for PCR assays due to its presence in multiple copies within the genome, thereby enhancing the sensitivity of detection (16, 35). However, since the number of IS*1111* copies in the *C. burnetii* genome varies between strains, the direct quantification of the number of organisms within a sample using this target is more complicated (17). A reliable PCR-based method for the detection and quantification of *C. burnetii* DNA therefore should include at least one single-copy marker for quantification and a multicopy target (e.g., IS*1111*) for enhancing the sensitivity of detection, preferentially in multiplex format and including an internal control. Most *C. burnetii* PCR assays have been designed as singleplex assays. Multiplexing PCR detection offers several advantages, including the reduction of sample volume and handling time (reducing the analysis time, cost, and opportunities for laboratory contamination). Also, false-negative results can be reduced through the coamplification 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 IS*1111* repetitive element (23, 31). The amplification of multiple signature sequences per organism also will 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 (19, 20).

In the current study, we describe the design and application of a multiplex qPCR assay for *C. burnetii* to investigate potential environmental and veterinary sources for human *C. burnetii* infection during a large Q fever outbreak in the Netherlands in 2008.

MATERIALS AND METHODS

Design of multiplex primers and hydrolysis probes for a multiplex qPCR assay. Signature sequences for the reliable detection of *C. burnetii* DNA were selected based on previous literature and sequences available in public databases. Targets selected are the isocitrate dehydrogenase gene (*icd*), an outer membrane protein-coding gene (*com1*), and a multicopy insertion element (IS*1111*). The *B. thuringiensis cry1b* plasmid gene was used as a signature sequence for the detection of DNA from this organism's spores, which were spiked into samples to serve as controls for both DNA extraction and PCR amplification. *Bacillus thuringiensis* spores are highly refractory, and successful DNA extraction from these spores will ensure DNA extraction from other biological structures (10, 13).

Sequences retrieved from NCBI/EMBL were organized and aligned using the software package Kodon (Applied Maths, Ghent, Belgium). Comprehensive sequence alignments were made by performing BLAST searches from the selected targets to make sure all available sequence homologues were included in the alignments. For each target, a consensus sequence was generated with an acceptation level of 100% for consensus determination, and the consensus sequence displayed all mutations.

Oligonucleotides for multiplex qPCR assays and for conventional PCR assays were designed using the software package Visual Oligonucleotide Modeling Platform version 6 (Visual OMP 6; DNA software Inc., Ann Arbor, MI). The design strategy for a multiplex qPCR assay was as follows. First, a hydrolysis probe and primer set were designed as the *B. thuringiensis* internal controls. For each of the three selected signature sequences for *C. burnetii*, a hydrolysis probe was designed, followed by the design of the corresponding primer set. Oligonucleotides that were calculated by the design software first were checked against the consensus alignment to exclude designs not covering all sequence variants and then were evaluated using the simulation module of Visual OMP 6. Finally, BLAST analysis was performed to confirm specificity for *C. burnetii*.

Multiplex qPCR and conventional PCR. Oligonucleotides were synthesized by Biolegio (Nijmegen, the Netherlands). Oligonucleotide sequences and labeling are detailed in Table 1. Dyes were coupled to the 5′ end and quenchers to the 3′ end, with the exception of a black hole quencher 2 (BHQ-2) coupled to the T at position 13 of probe Tqpro_Bt. All qPCRs were carried out in a final volume of 20 μl containing IQ Multiplex Powermix (Bio-Rad), 200 nM primers, and 100 to 300 nM hydrolysis probes. Probe concentrations were optimized for each batch to yield a comparable fluorescence for each dye. For the current study, concentrations were 100, 200, 300, and 300 nM for probes labeled with fluorescent dyes carboxyfluorescein (FAM), JOE, CFR590 and Cy5, respectively. PCR amplification conditions were the following: 95°C for 5 min, 50 thermocycles at 95°C for 5 s and 60°C for 35 s, followed by a final incubation step at 50°C for 30 s. We included 3 μl of *C. burnetii* Nine Mile RSA phase I DNA template as the positive control or 3 μl H_2O as the negative control. Multiplex qPCR assays were carried out on a LightCycler 480 instrument (Roche Diagnostics Nederland B.V, Almere, the Netherlands), and analysis was performed on the instrument's software (release 1.5.0. SP3). Quantification cycle (Cq) values were calculated using the second derivative method. Color compensation was carried out according to the Roche LightCycler 480 manual. To investigate a reduction in amplification efficiency due to multiplexing PCRs, we compared singleplex and multiplex PCRs on genomic DNA (gDNA) from the *C. burnetii* Nine Mile RSA phase I strain. Five replicate reactions were carried out in singleplex format for each target and in multiplex format under identical reaction conditions. Differences in amplification efficiency were assessed by shifts in Cq values between multiplex and singleplex reactions for the same targets.

Conventional PCR was used to produce extended target amplicons from signature sequences using DNA of *C. burnetii* Nine Mile RSA phase I as the template. These extended target amplicons were used for investigating qPCR efficiency and the limit of detection (LOD) for the different targets. Primers were designed using Visual OMP 6 software, and amplification was carried out using the HotStarTaq master mix (Qiagen, Westburg, Germany) and 400 nM primers in a total reaction volume of 50 μl. Thermocycling conditions were the following: 95°C for 15 min, 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s, followed by a final step at 72°C for 10 min. We included 3 μl of *C. burnetii* Nine Mile RSA phase I DNA template. Conventional PCRs were carried out in a Px2 thermal cycler (Thermo Electron Corporation, Breda, the Netherlands). The verification of the size of all PCR products in this study was carried out on the Agilent 2100 Bioanalyzer instrument (Agilent Technologies Netherlands B.V., Amstelveen, the Netherlands) using the instrument's software (version B.02.07).

Titer of spore suspension used as internal control. Spores of *B. thuringiensis* were chosen as the internal control for DNA extraction and PCR amplification. Spore suspensions of *B. thuringiensis* strain ATCC 29730 were obtained from Raven Biological Laboratories (Omaha, NE). The amount of spores that needs to be added to samples to obtain suitable Cq values for this internal control was determined empirically for each stock spore suspension. Tenfold dilutions were made from the spore stock, and DNA was extracted from 50-μl portions of each dilution by using the NucliSens magnetic extraction kit (bioMérieux, France). The developed multiplex qPCR assay was used to determine the amount of spores required for a Cq value between 30 and 32.

Efficiency, reproducibility, and limit of detection. The characterization of qPCR performance was guided by the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (8). Standard curves for targets *icd*, *com1*, and IS*1111* were made using the extended target amplicons described above. PCR products for each of the three targets were purified using the Qiaquick PCR purification kit (Qiagen, Venlo, the Netherlands). The concentration of purified PCR products was measured using a Nanodrop 1000 (Isogen Life Science, De Meern, the Netherlands), and the number of copies per μl was calculated for each target from their known sizes and DNA concentrations.

Efficiency and reproducibility were calculated from the Cq values obtained from a 6-log linear dilution series from extended amplicon mixtures as templates. These mixtures contained all three target sequences, and triplicate measurements were obtained for each dilution.

To determine the LOD, a 2-fold dilution series of 10 concentrations was prepared. The dilution range included a concentration at which all reactions were positive and one at which all were negative. Eight replicates were measured from each dilution. This experiment was repeated three times in separate mul-

TABLE 1. Primers and hydrolysis probes for multiplex qPCR and conventional PCR

Primer or probe	Designation	Primer and probe sequences (5′–3′)	Product length (bp)
qPCR			
Target *icd*			
Forward primer	icdpri_f	GACCGACCCATTATTCCCT	139
Reverse primer	icdpri_r	CGGCGTAGATCTCCATCCA	
Probe	Tqpro_icd	CFR590-CGCCCGTCATGAAAAACGTGGTC-BHQ1	
Target *com1*			
Forward primer	compri_f	AAGCAATTAAAGAAAATGCAAAGAAATTAT	133
Reverse primer	compri_r	ACAGAATTCATGGCTTTGCAAT	
Probe	Tqpro_com	JOE-CACATTGATAATCGAAAAATTCAACCAATG-BHQ1	
Target IS*1111*			
Forward primer	IS1pri_f	CGCAGCACGTCAAACCG	146
Reverse primer	IS1pri_r	TATCTTTAACAGCGCTTGAACGTC	
Probe	Tqpro_IS1	FAM-ATGTCAAAAGTAACAAGAATGATCGTAAC-BHQ2	
Target c*ry1b*			
Forward primer	Btpri_f	GCAACTATGAGTAGTGGGAGTAATTTAC	132
Reverse primer	Btpri_r	TTCATTGCCTGAATTGAAGACATGAG	
Probe	Tqpro_Bt	Cy5-ACGTAAATACACTTGATCCATTTGAAAAG-BHQ2	
Conventional PCR			
Target *icd*			
Forward primer	icdtrg_f	CGGAGTTAACCGGAGTATCCA	738
Reverse primer	icdtrg_r	CCGTGAATTTCATGATGTTACCTTT	
Target *com1*			
Forward primer	comtrg_f	CCCTGCAATTGGAACGAAG	775
Reverse primer	comtrg_r	GTTCTGATAATTGGCCGTCGACA	
Target IS*1111*			
Forward primer	IS1trg-f	AGAATTTCTATTTTCAAAAAAAGGAGAAG	605
Reverse primer	IS1trg-r	CGGTTCAACAATTCGGTATACAAACAA	

tiplex qPCR runs, and the number of positive qPCR amplifications in each dilution was scored. The minimal target concentration that could be detected with 95% probability was calculated for each target by performing a probit analysis (37) using the statistical software package SPSS v.15.

Specificity and coverage of strain diversity. A DNA panel from nontarget bacterial species was used to validate the specificity of the developed multiplex qPCR assay. These nontarget organisms include *Bacillus thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *aizawai*, *B. thuringiensis* var. *galleriae*, *B. thuringiensis* Berliner 1915 AL (ATCC 10792), *B. cereus* Frankland 1887 AL (ATCC 14579), *B. cereus*, *B. mycoides* Flügge 1886 AL (ATCC 6462), *B. mycoides* (three isolates), *Yersinia pseudotuberculosis* (three isolates), *Y. agglumerans*, (three isolates), *Y. frederiksenii*, *Y. enterocolitica* (six isolates), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* (ATCC 15442), *Legionella pneumophila* serotype 1 (ATCC 33152), *L. bozemonii* (ATCC 33217), *L. longbeachae* (ATCC 33462), *L. micdadei* (ATCC 33218), *L. dumoffii* (ATCC 33279), *L. anisa* (ATCC 35292), *Rickettsia heilongjiangii*, *R. akari* (ATCC VR-148), *R. helvetica*, *R. typhi* (ATCC VR-142), *R. monacensis*, *R. africae*, *R. conorii* (ATCC VR-613), *R. honei*, and *R. prowazekii*.

Nontarget organisms, especially near relatives, such as *Legionella* species, could be differentiated as evidenced by the absence of amplification. The *in vitro* validation of *C. burnetii* strain coverage has become complicated, because obtaining *C. burnetii* material or even DNA is restricted for biosecurity reasons. At the moment of the experiments described in this study, DNA of *C. burnetii* strains available at RIVM was limited to three strains and isolates. DNA of *C. burnetii* strain Nine Mile RSA phase I and isolates from mouse spleen (EP3, Russia, 1958, *Apodemus flavicollis*), and tick (EP5, Slovakia, 1968, *Dermacentor marginatus*) were used in experiments to test the performance of the qPCR assay. In addition, the developed multiplex qPCR assay was tested in a ring trial for *C. burnetii* DNA detection, in which 10 DNA samples from various origins were compared among seven laboratories (14).

Sampling environmental and veterinary matrices for *C. burnetii* screening. The selection of farms, which were thought to be a potential source for human Q fever in their near vicinity, was conducted by various Municipal Health Services (GGD) in close collaboration with the Food and Consumer Product Safety Authority (VWA). Primarily commercial dairy goat farms in a highly Q fever-affected area in the Netherlands were selected, based on the criteria of (i) the emergence of human Q fever cases within their near vicinity, (ii) a history of abortions among goats before 2007, and (iii) a reported abortion percentage greater than 5% among goats in 2007 and 2008. In total, 29 farms were selected to investigate potential veterinary and environmental sources for *C. burnetii* infection during the Q fever epidemic in 2008. From these 29 farms, 3 were regarded as negative farms where no (or relatively very low levels) of *C. burnetii* DNA was to be expected. A random sample of animals was selected on each farm, and vaginal swabs were obtained from goats, sheep, or cattle using sterile cotton swabs (VWR International, The Netherlands). On a selection of farms, surface area swabs were obtained from horizontal (dust-accumulating) surfaces, such as windowsills or low stable compartment boundary walls. Surface area swabs were obtained by swabbing an Enviroswab (Biotrace International, United Kingdom) in a single motion across a length of two meters. Materials were transported to the laboratory at 10°C and stored at 4°C until processing within 1 month.

Sample processing and DNA extraction procedures. Environmental and veterinary samples were processed, and DNA was extracted using the NucliSens magnetic extraction kit (bioMérieux, France). Small modifications were made to the manufacturer's guidelines for DNA isolation from environmental and animal swabs. Surface area swabs and vaginal swabs were added to 10 ml of NucliSens lysis buffer, vortexed for 10 s, incubated for 10 min, and then removed. As an internal control for both DNA extraction and amplification, 50 μl of a *B. thuringiensis* spore suspension (1.2×10^5 spores) was added to each sample and to a blank sample (containing water). All samples were stored at room temperature for 1 h to complete lysis. From this point onwards, DNA isolation procedures were carried out according to the manufacturer's protocol.

RESULTS

Efficiency, reproducibility, and limit of detection. Tenfold independent serial dilutions, from purified target amplicons ranging between 10^2 and 10^7 target copies per reaction, were used to generate standard curves and calculate PCR amplification efficiencies and the reproducibility of the multiplex qPCR assay. Efficiencies were calculated for all four targets and to investigate reproducibility, and mean Cq values and standard deviations (SD) were calculated for each dilution

TABLE 2. Efficiency, reproducibility, and detection limit of the multiplex qPCR

Organism	Target	Efficiency[a] (%)	Reproducibility[a] (SD of Cq)	LOD[b] (copies/reaction)
C. burnetii	*icd*	98.9 ± 0.8	0.1	13.0 (7.9–41.0)
	com1	97.2 ± 1.5	0.2	10.6 (5.1–244.7)
	IS*1111*	98.2 ± 0.9	0.1	10.4 (6.2–35.9)
B. thuringiensis	*cry1b*	98.7 ± 0.7	0.2	Not determined

[a] Values represent the averages and standard deviations calculated from three replicate Cq measurements of six target concentrations ranging from 10^2 to 10^7 copies per reaction.

[b] Values displayed represent the lowest DNA concentrations at which 95% of the positive samples are detected, as calculated by using probit analysis. Shown between brackets are the 95% confidence limits of the calculated LODs.

(Table 2). Only at very low concentrations (10^1 target copies) near the limit of detection did the SD for *com1* exceeded 1 qPCR cycle.

The LOD, calculated by using probit analysis, was 13.0 copies per reaction for target *icd*, 10.6 for target *com1*, and 10.4 for target IS*1111* (Table 2). The highest Cq values obtained were 37.0 qPCR cycles for *icd*, 38.7 cycles for *com1*, and 39.0 cycles for IS*1111*.

A reduction in amplification efficiency due to multiplexing qPCRs was not observed. Using genomic DNA of the *C. burnetii* Nine Mile RSA phase I strain, we found an average difference of less than 1 qPCR cycle between the Cq values of multiplex and singleplex reactions for each target. For target *icd*, averaged Cq values were 26.3 ± 0.1 and 25.1 ± 0.1 for singleplex and multiplex reactions, respectively. For target *com1*, the average Cq values were 27.3 ± 0.1 for singleplex reactions and 26.3 ± 0.1 for multiplex reactions. Finally, for target IS*1111*, average Cq values for singleplex and multiplex reactions were 22.6 ± 0.1 and 22.2 ± 0.1, respectively.

Specificity and coverage of strain diversity. The specificity of the multiplex qPCR assay was investigated using a large panel of nontarget organisms, including six species of the closely related genus *Legionella*. No cross-reactivity with any of these species was observed. The qPCR assay amplified target sequences from all three *C. burnetii* strains that were tested: reference strain Nine Mile RSA phase I, a mouse spleen isolate, and a tick isolate. In addition, the multiplex qPCR assay was tested in a ring trial for *C. burnetii* DNA detection. Ten DNA samples, obtained from fetal fluids and placenta materials of ovine, caprine, and bovine origin, were compared between seven laboratories (14).

Due to the presence of multiple copies within the *C. burnetii* genome (17), the amplification of target IS*1111* is expected to occur before the amplification of the single-copy target *icd* or *com1*. This was reflected in our data from highly diluted samples where low levels of *C. burnetii* DNA were expected and from the ring trial. In samples in which all targets were amplified, the differences in Cq values between single-copy targets *icd* and/or *com1* and multicopy target IS*1111* was approximately 3 PCR cycles. Cq values for IS*1111* were always lower than those for *icd* and/or *com1*. Differences in Cq values between *icd* and *com1* were less than 1 qPCR cycle. Only when the Cq values for both single-copy targets reached their detection limit did this difference occasionally become larger. For instance, when highly diluted DNA of the *C. burnetii* Nine Mile RSA phase I strain was used, Cq differences between *icd* and *com1* of 1.3 ± 0.4 and 2.2 ± 0.6 qPCR cycles were observed for the two highest detectable dilutions.

Screening environmental and veterinary matrices for *C. burnetii* presence. The presence of *C. burnetii* DNA in veterinary (vaginal swabs) and environmental matrices (surface area swabs), obtained from 29 farms in 2008, was investigated and is summarized in Table 3 and visualized in Fig. 1. Samples can be categorized into four categories, with increasing *C. burnetii* DNA content: (1) negative, (2) IS*1111* positive, (3) IS*1111* plus *com1* or IS*1111* plus *icd* positive, and (4) IS*1111* plus *com1* plus *icd* positive. Samples which scored positive for two *C. burnetii* targets (IS*1111* plus *icd* or IS*1111* plus *com1*) are shown in separate columns in Table 3.

From the 29 farm locations, 300 vaginal swabs of goats, sheep, and cattle were obtained, of which 164 (55%) were scored as negative and 136 (45%) scored as positive for any of the three positive categories. Out of 68 surface area swabs, 31 (46%) were scored as negative and 37 (54%) were scored as positive for one, two, or all three *C. burnetii* targets.

The Cq value for samples positive for IS*1111* only was always considerably higher than that for samples where more than one target was amplified. On farms where both veterinary and environmental samples were screened and positive results were found (farms 1, 8, 10, 16, and 23), Cq values for target IS*1111* were lower for environmental samples than for veterinary samples. The differences between environmental and veterinary samples for the average Cq values for target IS*1111* ranged between 3.67 qPCR cycles on farm 10 and 9.84 qPCR cycles on farm 1. Finally, in veterinary and environmental samples the difference between the single-copy targets was less than 1 qPCR cycle.

qPCR inhibition by environmental matrices. The inhibition of qPCR was investigated by assessing the difference between the Cq of the internal control target *cry1b* in a sample and that in the control containing *B. thuringiensis* spores in water. The addition of 1.2×10^5 spores (50 μl diluted spore suspension) of *B. thuringiensis* to the DNA extraction resulted in an average Cq of 31.4 ± 1.2 qPCR cycles. Tenfold and 100-fold dilutions of samples were carried out when the internal control for a given sample exceeded this averaged Cq value or if no signals were obtained.

Vaginal swabs from goats, sheep, and cattle showed no qPCR inhibition, with Cq values for the internal control within the range of the positive control. In contrast, for some surface area swabs, separate qPCR runs failed to produce a signal for the internal control *cry1b* when undiluted DNA extracts were tested. For these samples, PCR amplification could be achieved only after 10- or 100-fold dilutions of the DNA extract. Occasionally, qPCR inhibition was indicated only by a shift in the Cq of internal target *cry1b*, resulting in a Cq value higher than that of the control and reaching a maximum at a Cq of 38. In all samples showing PCR inhibition, positive signals for the internal control target *cry1b* were obtained after dilution and, in many cases, for any of the three *C. burnetii* targets (Table 3).

To investigate the impact of qPCR inhibition on the different amplifications in the multiplex qPCR, DNA extraction was performed on a surface area swab which had previously tested

TABLE 3. Presence of *C. burnetii* DNA in veterinary and environmental samples obtained from 29 farms in the Netherlands in 2008[a]

Farm	Source	No. of samples	No. negative	No. positive for:			
				IS*1111*	IS*1111* + *icd*	IS*1111* + *com1*	IS*1111* + *com1* + *icd*
1	Goat	3				3	
1	Surface area swab	4				1	3
2	Cow	19	19				
3	Surface area swab	3	3				
4	Sheep and goat	21	19			2	
5	Goat	10		7	1		2
6	Sheep	5		3			2
7	Sheep and goat	12	9	3			
8	Goat	10			2	1	7
8	Surface area swab	9	1		2	1	5
9	Surface area swab	5					5
10	Goat	13	4	7		2	
10	Surface area swab	7	3	4			
11	Surface area swab	5		2		3	
12	Sheep and goat	12	9			3	
13	Sheep	10	10				
13	Surface area swab**	5 (5)	5 (5)				
14	Goat	4	4				
14	Surface area swab*	5 (5)	5 (5)				
15	Goat	19	9	6		4	
16	Sheep and goat	15	13	2			
16	Surface area swab*	8 (7)	6 (4)	1 (1)	1		
17	Goat	2	1	1			
17	Surface area swab	5	5				
18	Goat	3	2	1			
19	Goat	20	3	4		11	2
20	Goat	10		9	1		
21	Goat	10		4	4	1	1
22	Sheep	16	16				
23	Goat	10		7	1		2
23	Surface area swab**	9 (9)		3 (3)	3 (3)		3 (3)
24	Cow	10	4	6			
24	Surface area swab*	3 (2)	3 (2)				
25	Sheep and goat	22	22				
26	Goat	4	4				
27	Sheep	9	7	2			
28	Sheep	12	4	7	1		
29	Cow and sheep	19	5	11	2	1	

[a] Samples were 10-fold (*) or 100-fold (**) diluted if internal control *cry1b* in undiluted samples was not detected. Numbers in parentheses are the total numbers of diluted samples and the numbers of diluted samples per category.

negative for *C. burnetii* DNA but which showed strong qPCR inhibition. Genomic DNA of *C. burnetii* Nine Mile phase I and of the *B. thuringiensis* internal control was spiked into a dilution series of the PCR-inhibiting DNA extract obtained from the surface area swab. Complete inhibition was seen for undiluted samples and samples at up to 50-fold dilutions. At a 100-fold dilution, all four targets, including the internal control, were detected, and Cq values for *C. burnetii* DNA approached those of the control (Table 4).

DISCUSSION

The development of a new multiplex qPCR assay for *Coxiella burnetii* was motivated by the emerging human Q fever epidemics of 2007 and 2008 in the Netherlands. The use of the presented multiplex qPCR makes it possible to avoid false-positive and false-negative results due to the application of multiple target sequences for the same species. In addition, we can make a qualitative distinction between samples with a relatively low concentration of *C. burnetii* DNA (IS*1111* positive only), an intermediate concentration (IS*1111* plus *icd* or IS*1111* plus *com1* positive), and a high *C. burnetii* DNA content (IS*1111* plus *icd* plus *com1*) within a specific matrix. This way, the amplified single-copy (*icd* and *com1*) and multicopy (IS*1111*) targets are used not only to confirm *C. burnetii* DNA presence but also to qualitatively estimate the *C. burnetii* DNA content when calibration curves for quantification in complex matrices are not available. In general, within the intermediate *C. burnetii* DNA concentration category (IS*1111* plus *icd* or IS*1111* plus *com1*), farms showed positive results for both possible combinations of a single-copy target and a multicopy target. A larger number of samples was positive for IS*1111* plus *com1* (33 samples) than for IS*1111* plus *icd* (18 samples). This may be explained by the differences in the detection limit between the single-copy targets, which is lower for *com1* than for *icd*.

It is unknown how many IS*1111* copies are present in the

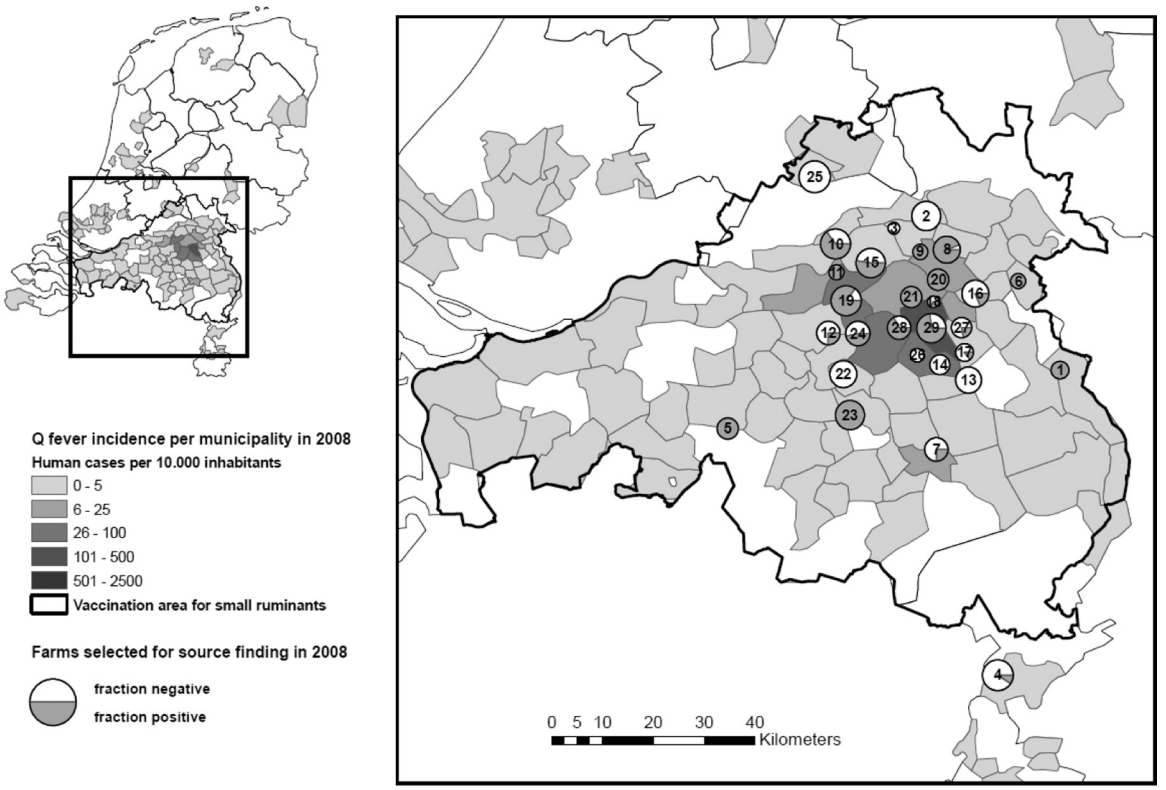

FIG. 1. Rural area in the Netherlands affected most by Q fever in the Netherlands in 2007 and 2008. The farm number and sample size per farm are indicated, and the proportion of positive samples (for any of the three *C. burnetii* targets) is indicated in gray and the proportion of negative samples in white in the pie chart. The number of human Q fever cases is classified into five groups and is normalized against the population density within four-digit postal code areas. The vaccination area for small ruminants, established in 2009, is indicated by a black line.

genome of the different *C. burnetii* types circulating in the Netherlands. The number of IS*1111* copies has been reported to range between 7 and 110 copies per isolate (17), which complicates the quantification of the number of organisms based on this target sequence only. Therefore, the inclusion of single-copy genes, like *icd* and *com1*, into the same assay is highly preferable over (singleplex) assays targeting the IS*1111* insertion element alone.

During the Q fever epidemics in the Netherlands, commercial dairy goat farms were implicated primarily as sources for human Q fever cases (15, 18, 34, 42). The primary transmission route of *C. burnetii* to humans and animals is thought to be through the inhalation of contaminated aerosols generated by animal products (15, 21, 27, 34). The highest concentrations of *C. burnetii* in veterinary matrices are found in birth materials, like amnion fluids and placentas (3, 24), and in lower quantities in milk (9, 30) and blood (40). Therefore, the most likely route for *C. burnetii* to enter the environment is by shedding through placenta materials and amnion fluids, for instance, during the lambing season. In our study, we focused primarily on vaginal swabs obtained from goats and sheep and surface area swabs obtained from dust-accumulating surfaces.

TABLE 4. Results of the qPCR-inhibiting experiment[a]

Dilution of qPCR-inhibiting DNA extract added to *C. burnetii* NM gDNA	qPCR inhibition (mean Cq [SD]) with:			
	cry1b	IS*1111*	*com1*	*icd*
No extract	NA	26.2 ± 0.1	28.9 ± 0.2	27.5 ± 0.1
Undiluted extract	ND	ND	ND	ND
3-Fold diluted	ND	ND	ND	ND
5-Fold diluted	ND	ND	ND	ND
10-Fold diluted	ND	ND	ND	ND
30-Fold diluted	ND	29.2*	31.5 ± 0.1	29.6 ± 0.4
50-Fold diluted	ND	26.8 ± 0.1	30.2 ± 0.1	28.4 ± 0.1
100-Fold diluted	32.5 ± 0.5	26.7 ± 0.1	29.9 ± 0.2	28.2 ± 0.1

[a] Various dilutions of a qPCR-inhibiting DNA extract, obtained from a surface area swab negative for *C. burnetii* DNA, were spiked with gDNA of the *C. burnetii* Nine Mile RSA phase I strain (NM). Mean Cq values obtained from two replicates show severe qPCR inhibition on *C. burnetii* targets *icd, com1*, IS*1111,* and *B. thuringiensis* internal control target *cry1b.* NA, not applicable; ND, not detected; *, single Cq value.

In 2008, three farms (2, 13, and 25) were not expected to be a potential source for human Q fever, because human Q fever incidence in their nearby vicinity was relatively low. This was reflected in both veterinary (vaginal swabs) and environmental samples (surface area swabs), since on these farms no samples scored positive for any of the three *C. burnetii* targets.

A large number of farms in the affected area, however, showed positive results for vaginal swabs and/or surface area swabs. Based on the Cq values for target IS*1111*, our findings showed that *C. burnetii* accumulates in relatively high concentrations onto surface areas compared to that for vaginal swabs obtained from animals. Due to the accumulation of dust over long periods of time, surface area swabs may give insight into

the presence of *C. burnetii* DNA on a farm over a longer period than that of vaginal swabs obtained from goats or sheep. Vaginal swabs obtained from animals provide information on shedding at the moment of sampling only. *Coxiella burnetii*-contaminated dust may be transported out of the stable during normal farming procedures and support the transmission of *C. burnetii* from stables to animals and humans via aerosolized, contaminated dust particles. It has been hypothesized in epidemiological studies in the Netherlands that dry weather conditions and increasing temperatures during spring and summer increase the formation of *C. burnetii*-contaminated dust particles, which then could be transported by wind (15, 34, 39).

On farms where positive results were found, the strongest PCR inhibition was found in some of the surface area swabs. Various environmental and veterinary matrices are known to inhibit PCR assays, which may lead to an underestimation of pathogen numbers (1, 12, 28). As an internal control, we used *B. thuringiensis* spores, which are among the most resistant of microbial structures. DNA extraction from such spores can be considered to be a reliable indicator for successful DNA extraction from other microbes, including *C. burnetii*. We used the signals from the *B. thuringiensis* gene *cry1b* not only to confirm successful DNA extraction but also to assess the potential inhibition of PCR amplification. Such amplification inhibition could be derived from an increase in the Cq values derived from the *B. thuringiensis* spores spiked in samples compared to those spiked in the water control.

DNA extraction efficiency may be different within and between veterinary and environmental matrices, which can be reflected by the absence or shift in Cq values for target *cry1b*. In addition, we used the Cq values for *cry1b* to get insight into qPCR inhibition by the two matrices. A considerable portion of *B. thuringiensis* and *C. burnetii* DNA in an inhibitory background was detected after diluting out the inhibitors in DNA extracts. After the dilution of the samples, Cq values for target *cry1b* reached the levels of diluted controls, which indicates that the shift or absence of signals for *cry1b* probably is better explained by qPCR inhibition than by a reduction in DNA extraction efficiency.

It has been reported that PCRs for different target sequences are not equally susceptible to inhibition by coextracted substances (12). We have shown that a large variation is present in the severity of qPCR inhibition between samples of a single environmental matrix. Therefore, our results are indicative and cannot be easily extrapolated to other veterinary and environmental matrices.

In our study, we have shown that complex veterinary and environmental matrices can be screened reliably for the presence of *C. burnetii* DNA by the inclusion of an internal control target and three *C. burnetii* targets in one multiplex qPCR assay. These data yield valuable insights into the occurrence and transmission of *C. burnetii*. Nevertheless, by using qPCR for detection the organism's viability is not assessed. An estimate of the portion of viable organisms in the veterinary and environmental samples is desirable, yet such data are very difficult to obtain for *C. burnetii*. It is difficult to grow the organism (due to its virulence and complicated growth requirements), which is particularly true when it is present in low numbers in complex matrices.

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