National Institute for Public Health
and the Environment
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B.J. van Rotterdam

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This investigation has been performed by order and for the account of the Food and Consumer Product Safety Authority (nVWA), within the framework of Livestock-borne Zoonoses, project 9.2.3.D: Coxiella

Abstract

Detection of *Coxiella burnetii* DNA in animal and environmental matrices on non-dairy sheep farms

During the spring and summer of 2007, 2008 and 2009, large Q fever outbreaks occurred in the Netherlands affecting a rural area in the southeast of the country. Prior to and during these outbreaks Q fever related abortion waves were reported on several dairy goat farms in the same region. As a result, primarily commercial dairy goat farms were implicated as potential sources for the emerging human Q fever cases in the Netherlands. However, in 2008 and 2009 a number of (non-dairy) sheep farms were identified where *C. burnetii* DNA was detected in both animal (vaginal swabs) and environmental (surface area swabs) matrices. In addition, in two epidemiological studies non-dairy sheep farms were implicated as the primary source for an emerging cluster of human Q fever cases in their near vicinity. Therefore, although less important in the recent epidemic, non-dairy sheep farms cannot be ruled out as potential source for human Q fever.
In the current study, we describe the presence of *C. burnetii* DNA in animal and environmental matrices obtained from two non-dairy sheep farms. We show that *C. burnetii* DNA content in surface area swabs from fences and drinking buckets and udder swabs from animals was consistently higher on farm B in comparison to farm A. This may be explained by the geographical locations of the farms, since farm B is located in a highly Q fever affected area (Noord-Brabant), while farm A is located in an area not affected by Q fever (Noord-Holland).
How these results are related to shedding of *C. burnetii* by the non-dairy sheep on these farms is not clear. No positive relationship was found between *C. burnetii* content in vaginal swabs and udder swabs. *Coxiella burnetii* contamination of sheep udders may be a result from excrements from the same animal, direct contact with other animals (or other contaminated surfaces), or a combination of these.

Keywords:
Coxiella burnetii, Q fever, Molecular detection, Sheep

Trefwoorden:
Coxiella burnetii, Q-koorts, Moleculaire detectie, Schapen

Contents

1 Potential sources for human Q fever in the Netherlands

1.1 Dairy goat farms as potential source for human Q fever

During the spring and summer of 2007, 2008 and 2009, large Q fever outbreaks occurred in the Netherlands, which affected 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 (32). Since most animals show no clinical symptoms of infection by *Coxiella burnetii*, aborting animals were tested using serology and were often diagnosed with Q fever. In addition, Q fever source-finding investigations conducted in 2008 and 2009 revealed that *C. burnetii* DNA was found on many commercial dairy goat farms (8, 13). As a result of these experimental findings, amongst many other indications, commercial dairy goat farms were primarily implicated as sources for the emerging human Q fever cases in the Netherlands (16, 27, 32).
In several studies, conducted primarily on dairy goat farms during outbreaks in 2008 and 2009, we showed that *C. burnetii* (DNA) is present in relatively high concentrations on dust accumulating horizontal surface areas when compared to vaginal swabs obtained from animals in the same stables (8, 11, 13).
Vaginal swabs obtained from animals provide information on shedding of the *C. burnetii* bacterium at the moment of sampling only, since it is well known that *C. burnetii* shedding by animals may vary over time (2, 3). In contrast, *C. burnetii* contaminated dust may accumulate over longer periods of time and can be transported out of the stable during normal farming procedures. Moreover, *C. burnetii* in the environment probably will remain infectious for months up to years. This supports the hypothesis that the major route of transmission of *C. burnetii* occurs via aerosolized contaminated dust particles (20, 24), which is also supported by epidemiological studies (16, 27, 30).

1.2 Non-dairy sheep farms as potential source for human Q fever

The main focus during the source-finding investigations in 2008 and 2009 was on dairy goat farms. In 2008, however, five out of 29 farms screened for *C. burnetii* presence were (non-dairy) sheep farms and on three of the farms *C. burnetii* DNA was present (10). One non-dairy sheep farm in the East of the country was thought to be involved in the emergence of a cluster of human Q fever cases in its near vicinity (36). In 2009, twelve out of 56 farms selected during source-finding investigations were non-dairy sheep farms, and on ten of these farms *C. burnetii* DNA was present (9). One of these non-dairy sheep farms in the South-east of the country was implicated as the primary source for an emerging cluster of human Q fever cases in its near vicinity in 2009 (33).
Farming procedures on (non-dairy) sheep farms differ in a number of ways from procedures on dairy goat farms. For instance, non-dairy sheep are generally kept outdoors on meadows, while dairy goats are mainly kept indoors.
In stables of dairy goat farms, the high concentration of goats, the accumulation of *C. burnetii,* and the more frequent occurrence of abortions are thought to play an important role in transmission of *C. burnetii* to humans. The major mode of transmission of *C. burnetii* from non-dairy sheep to humans in the Netherlands is not clear.
In the current study, we describe the presence of *C. burnetii* in animal and environmental matrices obtained from two non-dairy sheep farms.

2 Material and Methods

2.1 Selection of non-dairy sheep farms

The two non-dairy sheep farms investigated in this study are included in a larger human-veterinary integrated Q fever study (Q-VIVE), which is funded by ZonMw (Netherlands Organisation for Health Research and Development), VWS (Ministry of Health, Welfare and Sport), EL&I (Ministry of Economic Affairs, Agriculture and Innovation), and the Food and Consumer Product Safety Authority (nVWA). In this larger project, epidemiological aspects of Q fever are investigated in both humans and animals on goat, sheep and cattle farms.
The two selected non-dairy sheep farms were visited by an employee of the Food and Consumer Product Safety Authority (nVWA) on 20-09-2010 (farm A) and 27-09-2010 (Farm B) to collect samples from several animal and environmental matrices.
Farm A is located in the province of Noord-Holland in a part of the Netherlands not affected by Q fever. Sheep are kept on meadows nearby the farm, except during lambing season (March-June) when they are kept indoors in stables. Based on data from bulk milk (tank) monitoring, the nearest Q fever affected (goat) farm is located on about 5.5 km distance from this farm.
Farm B is located in Noord-Brabant in the area in the Netherlands most affected by Q fever during the epidemics in 2007, 2008, and 2009. Sheep are kept outdoors on meadows nearby the farm from April to December and are kept the rest of the year indoors in stables. The nearest Q fever affected (goat) farm is located on about 8.5 km distance from this farm.

2.2 Sampling procedures for animal and environmental matrices

On both farms, samples were obtained from both animal and environmental matrices. Animal samples were obtained from a subset of the sheep population. From 30 animals, both vaginal and udder swabs were obtained, and 5 manure droppings were collected from the meadow. In addition, environmental matrices were represented by surface area swabs and aerosols. Ten surface area swabs were obtained from drinking buckets and fences. Two aerosol samples were obtained from within the herd and four aerosol samples were collected on 500 m distance from the herd in all four wind directions (North, East, South, and West). Surface area swabs and vaginal swabs and udder swabs of animals were taken using sterile cotton swabs (VWR International, the Netherlands). Manure samples were collected by adding sheep droppings to Phosphate-buffered Saline (PBS) in 50 ml Greiner tubes (Greiner Bio-one, the Netherlands), using a 1:2 ratio of manure and PBS. Aerosol samples were collected by using a Sartorius MD8 Airport. Aerosols were captured on nitrate-cellulose filters (pore size 8 µm), by sampling 500L of air using the pre-installed program of 10 minutes sampling with an air flow of 50L per minute. After collection, all obtained environmental and animal samples were transported to the laboratory, and stored at -20 ºC.

2.3 Sample processing and DNA extraction procedures

Animal and environmental samples were processed and DNA was extracted using the NucliSENS Magnetic Extraction kit (Biomerieux, France).
Small modifications were made to the manufacturer's guidelines for DNA extraction from animal and environmental matrices. Surface area swabs and vaginal swabs were added to 10 ml of NucliSens lysisbuffer, vortexed for 10 sec, incubated for 10 minutes and then removed. Manure samples (suspension of 1:2 ratio of manure and PBS) were homogenized for about 2 hours on a rotating tube holder at 10 rpm. Greiner tubes were centrifuged (Varifuge 3.2RS, Heraeus) at 2000 rpm for 10 minutes. The supernatant was transferred to a new Greiner tube, and 1 ml of supernatant was added to 10 ml of NucliSens lysisbuffer. Cellulose Nitrate filters, used in aerosol sample collection, were placed in petri dishes and submerged in 10 ml NucliSens lysisbuffer. Petri-dishes were then placed on a horizontal shaker for 2 hours at 50 rpm, after which the filters were removed. The lysisbuffer was transferred from the Petri dishes to 15 ml Greiner tubes.
As internal control, 50 µl of a *B. thuringiensis* spore suspension (1.2×10^5 spores) was added to each sample. All samples were placed at room temperature for one hour to complete lysis. From this point onwards, DNA isolation procedures were carried out according to the manufacturer's protocol.

2.4 Detection of *C. burnetii* DNA by multiplex real time PCR (qPCR)

The set-up of a modified multiplex real time PCR assay (qPCR) for *C. burnetii* was described elsewhere (12). One single copy target (*icd*) was removed from the assay since one single copy target (*com1*) proved to be sufficient for screening purposes. For target sequences of *com1* and *IS1111*, new primers and new (hydrolysis) probes were designed using software package Visual OMP 6. The qPCR assays were carried out on a LightCycler 480 Instrument (Roche Diagnostics Nederland B.V, Almere, the Netherlands). For all qPCR experiments we included positive and negative (no template) controls and each sample was tested undiluted, and in 10-fold and 100-fold diluted samples. All dilutions were tested in triplicate. Analysis of the data was performed on the software provided by Roche (LightCycler 480 Software release 1.5.0. SP3).

2.5 Quantification of *C. burnetii* DNA

The aspects regarding the quantification of *C. burnetii* (DNA) are described extensively in the appendix.
Due to its presence in multiple copies within the *C. burnetii* genome (17), amplification of target *IS1111* is expected to occur before amplification of the single copy target *com1*. This leads to a very sensitive detection of *C. burnetii* DNA in comparison to detection using single copy genes like *com1*. However, it is unknown how many *IS1111* copies are present in the genome of the different *C. burnetii* types circulating in the Netherlands. The number of *IS1111* copies has been reported to range between 7 and 110 copies per isolate (17), which complicates the quantification of the number of organisms when based on this target sequence only.
Therefore, to make a qualitative distinction between low and high levels of *C. burnetii* DNA, samples are scored as *IS1111*-positive (low *C. burnetii* DNA content), or *com1* and *IS1111*-positve (high *C. burnetii* DNA content).

Samples were scored as negative when none of the two *C. burnetii* targets showed a positive signal, whereas the internal control *cry1* showed a positive result.
This way, the amplified single copy (*com1*) and multicopy (*IS1111*) targets were used not only to confirm *C. burnetii* presence, but also to qualitatively estimate the *C. burnetii* DNA content when calibration curves for quantification in complex matrices are not available.
In addition, within the classes of low and high levels of *C. burnetii* DNA content, the level of *C. burnetii* DNA content is indicated by Cq values for targets *com1* and *IS1111*. Cq values represent the PCR cycles at which amplified DNA of targets *com1* and *IS1111* is detected. Therefore, samples with high *C. burnetii* DNA load show lower Cq values for targets *com1* and *IS1111* than samples with low *C. burnetii* DNA content.
Since information on Cq values for single copy target *com1* is very limited, the assessment of *C. burnetii* DNA is based primarily on Cq values for target *IS1111*, with the above mentioned reservations on copy numbers per *C. burnetii* strain taken into account. Therefore, the quantity of *C. burnetii* DNA is indicated by Cq values for each matrix, and is not expressed as the number of *C. burnetii* organisms present. A more extensive discussion on the aspects of *C. burnetii* quantification is described in appendix 7.2.

3 Detection of *C. burnetii* DNA on non-dairy sheep farms

The presence of *C. burnetii* DNA in vaginal swabs, udder swabs, manure, surface area swabs, and aerosols obtained from 2 non-dairy sheep farms (A & B) is summarised in Table 1, and visualized in Figures 1 & 2. The results for vaginal swabs and udder swabs of individual animals for both farms are given in supplemental Tables 2 & 3 in Appendix 7.1.

3.1 Comparing *C. burnetii* DNA content in matrices between farms

Overall, on farm A, 32 samples (41%) were found positive for *C. burnetii* DNA and on farm B 72 samples (92%). The highest *C. burnetii* DNA content, based on the Cq values for target *IS1111*, was found on farm A in DNA extracts from vaginal swabs, and on farm B in DNA extracts from udder swabs. The lowest *C. burnetii* DNA content (highest Cq values) were found on farm A in DNA extracts obtained from surface area swabs, and on farm B in DNA extracts from aerosol samples.
Between farms, *C. burnetii* DNA content in udder swabs and surface area swabs was significantly higher on farm B than on farm A ($p<0.01$[1]). *C. burnetii* DNA content in vaginal swabs ($p= 0.887$[1]) and aerosol samples ($p= 0.800$[1]) was not significantly different between the two farms.

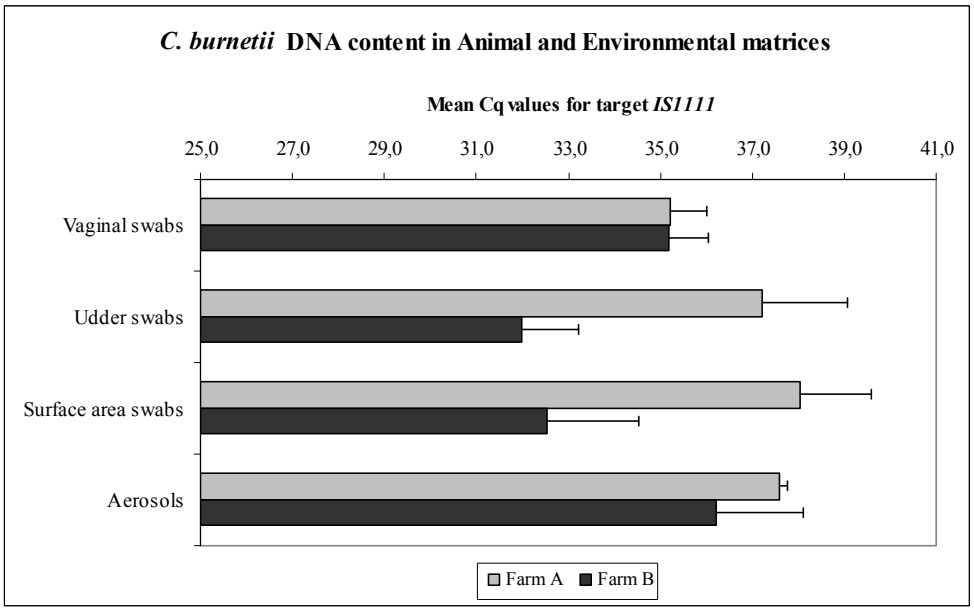

Figure 1. Between farm comparisons of *C. burnetii* DNA content in four different matrices obtained from two non-dairy sheep farms. High Cq values for target *IS1111* indicate low *C. burnetii* DNA content.

[1] Non-parametric Mann-Whitney U test

Table 1. *C. burnetii* DNA content in various animal and environmental matrices on two non-dairy sheep farms.

Farm	Description	Sample size	Positive samples (%)	No *C. burnetii* DNA load (negative)	Low *C. burnetii* DNA load (*IS1111*-positive)	High *C. burnetii* DNA load *(IS1111 + com1 positive)*	Cq_{com1} (SD)	Cq_{IS1111} (SD)
A	Vaginal swab	30	66,7	10	20			35.2 (0.8)
	Udder swab	30	26,7	22	7	1	38,5*	37.2 (1.9)
	Surface area swab	8	25,0	6	2			38.1 (1.5)
	Aerosols (Meadow)	2	50,0	1	1			37,5*
	Aerosols (500 m)	3	33,3	2	1			37,7*
	Manure	5	0,0	5				
B	Vaginal swab	30	96,7	1	25	4	37.7 (0.4)	35.2 (0.9)
	Udder swab	30	100,0		4	26	35.7 (0.8)	32.0 (1.2)
	Surface area swab	10	100,0		2	8	36.0 (1.3)	32.6 (2.0)
	Aerosols (Meadow)	2	100,0		2			35.1 (1.0)
	Aerosols (500 m)	4	25,0	3	1			38,3*
	Manure	2	0,0	2				

*Single values

3.2 Comparing *C. burnetii* DNA content in matrices within farms

Within farms, a number of matrices differed significantly in *C. burnetii* DNA content. On farm A, *C. burnetii* DNA content in vaginal swabs was significantly higher than in surface area swabs ($p<0.01$[1]). On farm B this pattern was reversed, with a significantly higher *C. burnetii* DNA content in surface areas swabs compared to vaginal swabs ($p<0.01$[1]). In addition, *C. burnetii* DNA content in udder swabs was significantly higher than in vaginal swabs on farm B ($p<0.01$[2]). On farm A, no significant difference was found between udder swabs and vaginal swabs ($p=0.345$[2]).
Finally, we tested whether there was a positive relationship between *C. burnetii* DNA content in vaginal swabs and udder swabs. On both farms no positive correlation was found between *C. burnetii* content in vaginal swabs and udder swabs ($p=0.285$[3] and $p=0.675$[3] for farms A and B respectively).

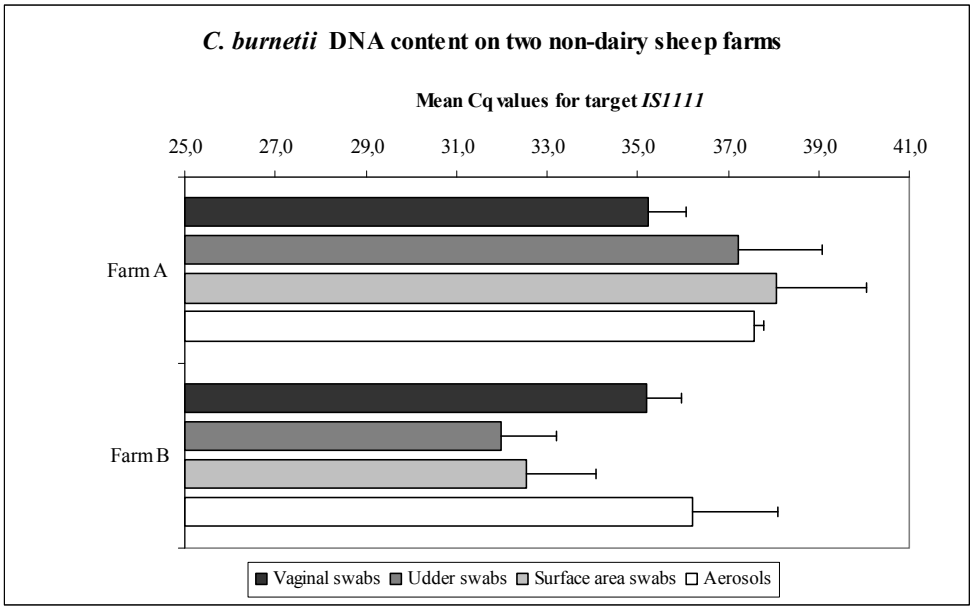

Figure 2. Within farm comparison of *C. burnetii* DNA content on two non-dairy sheep farms, obtained from four different matrices. High Cq values for target *IS1111* indicate low *C. burnetii* DNA content.

[2] Non-parametric Wilcoxon signed rank tests
[3] Speaman's rank order correlation

4 Discussion

The results of this study on two non-dairy sheep farms show that *C. burnetii* DNA is present in both animals and environmental matrices within the sheep herd and in the direct surroundings of the herd. *C. burnetii* DNA content in surface area swabs and udder swabs was found consistently higher on farm B in comparison to farm A. This may be explained by the geographical location of the two farms. Farm B is located in a highly Q fever affected area (Noord-Brabant), while farm A is located in an area not affected by Q fever (Noord-Holland).
How these results are related to shedding of *C. burnetii* by the non-dairy sheep on these farms is not clear. On both farms, no positive relationship was found between *C. burnetii* content in vaginal swabs and udder swabs. This result may be explained by the difference of the matrices examined and/or the moment of sampling. Vaginal swabs provide information on shedding of *C. burnetii* by animals at the moment of sampling only. Udder swabs may provide information on shedding over a longer period of time. *Coxiella burnetii* excreted via vaginal mucus, manure, and urine may contaminate and accumulate on udders over prolonged periods of time. On farm A, no significant difference in *C. burnetii* DNA content was found between vaginal swabs and udder swabs. This indicates that at the moment of sampling the sheep were shedding *C. burnetii* (20 out of 30 animal samples), and contamination of udders is therefore very likely.
On farm B, however, *C. burnetii* DNA content in udder swabs is consistently higher than in vaginal swabs. This may indicate that at the moment of sampling, excretion of *C. burnetii* by the animals was very low. This may explain the lack of a positive correlation between *C. burnetii* DNA content in vaginal swabs and udder swabs on both farms A and B.
Coxiella burnetii contamination of sheep udders is most probably a result from excrements from the same animal. However, other possibilities, like direct contact with other animals or other contaminated surfaces, or a combination thereof cannot be ruled out.

5 Conclusions

- *C. burnetii* DNA is present on the selected non-dairy sheep farms.
- The number of positive samples on farm B is higher than on farm A.
- *C. burnetii* DNA content in surface area swabs and udder swabs was significantly higher on farm B than on farm A.
- On farm A, *C. burnetii* DNA content in vaginal swabs was significantly higher than in surface area swabs.
- On farm B, *C. burnetii* DNA content in udder swabs and surface area swabs was significantly higher than in vaginal swabs.
- On both farms, no clear relationship was found in *C. burnetii* DNA content between vaginal swabs and udder swabs.

6 Recommendations

To obtain a better understanding of the relationship between animal and environmental matrices concerning the *C. burnetii* DNA content, we propose time series analyses of *C. burnetii* DNA content in vaginal swabs and udder swabs of the same animals, and surface area swabs obtained from the direct surroundings of the herd.
In addition, the viability of *C. burnetii* organisms present in animal and environmental matrices cannot be assessed using the current qPCR assay. We propose that the assessment of viability of *C. burnetii* organisms will be an important aspect of the 'VWA kennisvraag' Coxiella in 2012. We propose the setup of a relatively new approach to distinguish between viable and dead *C. burnetii* organisms using a technique referred to as viability PCR.
Finally, we have added a document in the appendix in which we describe the different aspects for the quantification of *C. burnetii* DNA in general and by using our multiplex qPCR for *C. burnetii* in particular. These aspects have to be addressed before an accurate quantification of the number of *C. burnetii* organisms in animal and environmental matrices can be achieved.

Acknowledgements

We would like to thank veterinarian Roel Paauwe of the Food and Consumer Product Safety Authority for his efforts in sampling of the farms, and the sheep farmers for their cooperation.

7 Appendix

7.1 Results of individual animals for two non-dairy sheep farms

Table 2. Results for vaginal and udder swabs for individual animals of farm A.

Sample Code	Animal nr.	Description	Mean Cq_{com1}	SD_{com1}	Mean Cq_{IS1111}	SD_{IS1111}	*C. burnetii* DNA content
1	1869	Udder swab	38,0		36,6	0,4	Udder swab > Vaginal swab
		Vaginal swab			36,8		
2	1546	Udder swab			34,3		Udder swab > Vaginal swab
		Vaginal swab			34,9		
3	1607	Udder swab					Udder swab = Negative
		Vaginal swab			33,5	2,3	
4	2069	Udder swab					Negative
		Vaginal swab					
5	1613	Udder swab			36,4		Udder swab < Vaginal swab
		Vaginal swab			34,3		
6	1881	Udder swab					Udder swab = Negative
		Vaginal swab			34,9		
7	1977	Udder swab					Udder swab = Negative
		Vaginal swab			35,8	0,3	
8	1941	Udder swab					Udder swab = Negative
		Vaginal swab			34,7	0,3	
9	1674	Udder swab					Udder swab = Negative
		Vaginal swab			35,0	0,3	
10	1895	Udder swab					Udder swab = Negative
		Vaginal swab			35,3	0,7	
11	1974	Udder swab					Udder swab = Negative
		Vaginal swab			34,6	0,5	
12	1604	Udder swab					Udder swab = Negative
		Vaginal swab			35,5	0,3	
13	ZN	Udder swab			36,4		Udder swab < Vaginal swab
		Vaginal swab			36,0	0,0	
14	1363	Udder swab			36,8	1,2	Udder swab < Vaginal swab
		Vaginal swab			35,8		
15	1912	Udder swab					Udder swab = Negative
		Vaginal swab			34,5		
16	1165	Udder swab					Udder swab = Negative
		Vaginal swab			35,7		
17	geel	Udder swab					Negative
		Vaginal swab					
18	1860	Udder swab					Negative
		Vaginal swab					
19	1947	Udder swab					Udder swab = Negative
		Vaginal swab			35,0	0,1	
20	1806	Udder swab					Negative
		Vaginal swab					
21	231	Udder swab					Negative
		Vaginal swab					
22	232	Udder swab			39,9	0,9	Vaginal swab = negative
		Vaginal swab					
23	raka ZN	Udder swab			37,8	0,7	Vaginal swab = negative
		Vaginal swab					
24	1808	Udder swab			39,6		Vaginal swab = negative
		Vaginal swab					
25	1813	Udder swab					Udder swab = Negative
		Vaginal swab			35,0	0,0	
26	36	Udder swab	39,0				Udder swab = Negative
		Vaginal swab			35,5	0,2	
27	73908	Udder swab					Udder swab = Negative
		Vaginal swab			35,2	1,5	
28	73983	Udder swab					Negative
		Vaginal swab					
29	2075	Udder swab					Negative
		Vaginal swab					
30	73985	Udder swab					Udder swab = Negative
		Vaginal swab			36,5	0,2	

Table 3. Results for vaginal and udder swabs for individual animals of farm B.

Sample Code	Animal nr.	Description	Mean Cq_{com1}	SD_{com1}	Mean Cq_{IS1111}	SD_{IS1111}	*C. burnetii* DNA content
1	173	Udder swab	35,8		31,8	0,2	Udder swab > Vaginal swab
		Vaginal swab			34,2	0,3	
2	24	Udder swab	36,6	0,1	33,1	0,1	Udder swab > Vaginal swab
		Vaginal swab			35,5		
3	76	Udder swab	36,4		32,0	0,1	Udder swab > Vaginal swab
		Vaginal swab			34,0		
4	104	Udder swab	35,0	0,1	31,2	0,0	Udder swab > Vaginal swab
		Vaginal swab			36,0	0,5	
5	363	Udder swab	35,6	0,2	30,0	0,1	Vaginal swab = Negative
		Vaginal swab					
6	307	Udder swab	36,7	0,1	32,8	0,3	Udder swab > Vaginal swab
		Vaginal swab			35,5	0,1	
7	335	Udder swab			32,7	0,1	Udder swab > Vaginal swab
		Vaginal swab			35,4	0,5	
8	354	Udder swab	34,5		31,2	0,1	Udder swab > Vaginal swab
		Vaginal swab			33,6	0,1	
9	91	Udder swab	36,0	0,2	31,9	0,4	Udder swab > Vaginal swab
		Vaginal swab			35,7	0,6	
10	2276	Udder swab	34,4	0,7	32,7	0,2	Udder swab > Vaginal swab
		Vaginal swab	38,1		35,5	1,2	
11	422	Udder swab	35,9		32,1	0,2	Udder swab > Vaginal swab
		Vaginal swab			36,1	0,1	
12	299	Udder swab	36,3	0,3	32,8	0,0	Udder swab > Vaginal swab
		Vaginal swab	37,9		34,6	0,3	
13	311	Udder swab	36,4	0,3	31,6	0,2	Udder swab > Vaginal swab
		Vaginal swab			35,3	0,1	
14	368	Udder swab	35,5		33,7	0,3	Udder swab > Vaginal swab
		Vaginal swab			34,3	0,2	
15	359	Udder swab	36,4	0,3	32,0	0,3	Udder swab > Vaginal swab
		Vaginal swab			34,1	0,1	
16	323	Udder swab	35,9		32,4	0,2	Udder swab > Vaginal swab
		Vaginal swab			36,4	0,3	
17	298	Udder swab	35,1	0,7	31,4	0,2	Udder swab > Vaginal swab
		Vaginal swab			36,2	0,4	
18	213	Udder swab			32,7	0,2	Udder swab > Vaginal swab
		Vaginal swab			34,2	0,3	
19	2428	Udder swab	34,6	0,7	29,6	0,1	Udder swab > Vaginal swab
		Vaginal swab			35,4	0,3	
20	271	Udder swab	35,8		32,7	0,1	Udder swab > Vaginal swab
		Vaginal swab			36,9	0,2	
21	3841	Udder swab			33,8	0,2	Udder swab > Vaginal swab
		Vaginal swab			34,2	0,4	
22	2287	Udder swab			33,7	0,2	Udder swab > Vaginal swab
		Vaginal swab	37,8		34,4	0,3	
23	405	Udder swab	36,1	0,8	30,4	0,2	Udder swab > Vaginal swab
		Vaginal swab			36,8	0,2	
24	159	Udder swab	34,4	0,3	29,0	0,1	Udder swab > Vaginal swab
		Vaginal swab			35,0	0,3	
25	656	Udder swab	37,3		33,8	0,2	Udder swab > Vaginal swab
		Vaginal swab			35,5	0,2	
26	136	Udder swab	35,9	0,2	32,4	0,1	Udder swab > Vaginal swab
		Vaginal swab			35,6	1,1	
27	180	Udder swab	36,0	0,2	31,6	0,1	Udder swab > Vaginal swab
		Vaginal swab			35,1	0,2	
28	69	Udder swab	35,8		32,7	0,2	Udder swab > Vaginal swab
		Vaginal swab	37,1	1,0	35,0	1,0	
29	74	Udder swab	33,9		31,2	0,2	Udder swab > Vaginal swab
		Vaginal swab			35,0	1,8	
30	404	Udder swab	35,8	0,6	30,3	0,2	Udder swab > Vaginal swab
		Vaginal swab			35,3	1,4	

7.2 Detection & quantification of *C. burnetii* by multiplex qPCR

7.2.1 PCR assays for the detection of C. burnetii DNA

Several PCR based diagnostic assays have been developed for the detection of *C. burnetii* DNA and have been used primarily for clinical samples (21, 29, 34). More recently, other types of PCR assays, like nested PCR (31, 35), and qPCR (4, 6, 17) have been developed, sometimes in combination with high-throughput capabilities (23). 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 the isocitrate-dehydrogenase gene (i*cd*), 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 (17, 28). 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 more complicated (8, 17). A reliable PCR based method for detection and quantification of *C. burnetii* DNA should therefore include at least one single copy marker for quantification and a multi-copy target (e.g. *IS1111*) for enhancing 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 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, and 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 (22, 26). 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 (18, 19).

Within the 'VWA Kennisvraag' for 2011, part 9.2.3D-6 is reserved for a comparison of the available qPCR assays for *C. burnetii* (both in theory and practice). This study will be a combination of peer reviewed literature and data obtained from national and international ring trials for *C. burnetii* detection using (q)PCR assays. The results of this study will be reported in a RIVM report in December of 2011, and together with this document will be the basis for an inventory on knowledge gaps in the quantification of *C. burnetii* DNA in animal and environmental matrices.
In the next paragraphs, the current methods used by RIVM for qualitative and quantitative assessment of *C. burnetii* DNA will be outlined. In addition, a number of important aspects will be discussed, which may be the basis for further research.

7.2.2 *Multiplex qPCR and assessment of C. burnetii DNA content*

The design and performance of a novel multiplex real time PCR assay (qPCR) for detection of *C. burnetii* DNA in animal and environmental matrices is described elsewhere (7). This assay was modified by removing one single copy target (*icd*), as one single copy target (*com1*) proved to be sufficient for screening purposes. In addition, the sensitivity of the assay was improved, by the development of new primers and (hydrolysis) probes for targets *com1* and *IS1111*, using software package Visual OMP 6 (12). The design and qPCR performance was guided by the MIQE guidelines (7) and tested in both international and national ring trials for the detection of *C. burnetii* in animal and environmental samples. The first ring trial was facilitated by the Veterinary Laboratories Agency, Weybridge, Addlestone, Surrey, United Kingdom (VLA), and results were published (15). The second ring trial was facilitated by RIVM-LZO and results were published in a RIVM report (5).

7.2.3 *Qualitative assessment of C. burnetii DNA*

Due to its presence of multiple copies within the *C. burnetii* genome, amplification of target *IS1111* is expected to occur before amplification of the single copy target *com1*.
This leads to a very sensitive detection of *C. burnetii* DNA in comparison to detection using single copy genes like *com1*. However, it is unknown how many *IS1111* copies are present in the genome of the different *C. burnetii* types circulating in the Netherlands. The number of *IS1111* copies has been reported to range between 7 and 110 copies per isolate, which complicates the quantification of the number of organisms when based on this target sequence only (17). However, a qualitative distinction between low and high levels of *C. burnetii* DNA can be made using the presence of different targets. Samples can be categorised as low in *C. burnetii* DNA content, when only target *IS1111* shows positive results. Samples can be categorised as high in *C. burnetii* content, when both targets *com1* and *IS1111* show positive results. Subsequently, samples can be scored as negative when none of both *C. burnetii* targets showed a positive signal, whereas the internal control *cry1* showed a positive result. This way, the amplified single copy (*com1*) and multicopy (*IS1111*) targets were used not only to confirm *C. burnetii* presence, but also to qualitatively estimate the *C. burnetii* DNA content when calibration curves for quantification in complex matrices are not available.

7.2.4 *Quantitative assessment of C. burnetii DNA*

The quantification of *C. burnetii* DNA can be complicated not only by the target of choice, but also by a phenomenon referred to as qPCR inhibition. Various environmental and animal matrices are known to inhibit PCR assays, which may lead to underestimation of pathogens present in these matrices (1, 14, 25). For *C. burnetii*, a method often used to correct for qPCR inhibition is to obtain standard curves from potentially qPCR inhibiting matrices. For instance, known concentrations of *C. burnetii* DNA are spiked into goat milk and the Cq values obtained are compared to Cq values obtained from standard curves containing only *C. burnetii* DNA. (Personal communication by Piet Vellema, Animal Health Service). This way, the level of qPCR inhibition can be assessed for a particular matrix, in this case goat milk. An important assumption using this method is that in further quantification of *C. burnetii* DNA qPCR inhibition is the same for each sample and is constant over time for that particular matrix.

In our experience, however, qPCR inhibition can vary significantly between samples of the same matrix.
In an EL&I funded project in 2010, and in collaboration with the Central Veterinary Institute, we used a method in which qPCR inhibition was assessed in individual manure samples on basis of an internal control. As internal control for DNA extraction and qPCR amplification we use *Bacillus thuringiensis* spores, which are among the most resistant of microbial structures. DNA extraction from *B. thuringiensis* spores can therefore be considered as a reliable indicator for successful DNA extraction from other microbes, such as *C. burnetii*. In addition, the severity of qPCR inhibition can be assessed using the internal control by comparison of Cq values obtained from inhibiting environmental samples ($Cq_{cry1\ sample}$) to Cq values obtained from a positive control containing only *B. thuringiensis* DNA ($Cq_{cry1\ p.c}$). This can be achieved using the following formula:

$$\Delta Cq_{cry1} = Cq_{cry1\ sample} - Cq_{cry1\ p.c}$$

The ΔCq_{cry1} is a measure for the level of qPCR inhibition in a particular sample. The value of ΔCq_{cry1} can be subtracted from the Cq values obtained for *C. burnetii* target sequences *com1* ($Cq_{com1\ sample}$) and *IS1111* ($Cq_{IS1111\ sample}$). This way, a correction in Cq values can be obtained for qPCR inhibition. However, an important assumption using this method is that qPCR inhibition affects all three targets, (*com1*, *IS1111*, and *cry1*) to the same extent.
In addition, to investigate the impact of qPCR inhibition on both the internal control target *cry1* and *C. burnetii* targets *com1* and *IS1111* targets amplified during multiplex qPCR, DNA extraction was performed on a sample previously tested negative for *C. burnetii* DNA, but which showed strong qPCR inhibition (8). Genomic DNA of *C. burnetii* and of the *B. thuringiensis* internal control were spiked into a dilution series of the PCR inhibiting DNA extract obtained from that sample. After qPCR, differences in PCR inhibition were assessed by comparing Cq values for all three targets in the dilutions. Results were not conclusive and minor differences were observed between Cq values for the internal control (*cry 1*) and *C. burnetii* targets (*com1* and *IS1111*). The effect of qPCR inhibiting substances on both the *C. burnetii* targets *com1* and *IS1111* and internal control target *cry1* have to be studies in more detail, before it can be used to screen for presence for *C. burnetii* DNA in animal and environmental matrices known to inhibit qPCR.

7.3 Important aspects for quantitative assessment of *C. burnetii* DNA

As described above, current methods have different assumptions, which may affect the accuracy of the quantification of *C. burnetii* DNA. The first method, using standard curves spiked with potentially qPCR inhibiting matrices, has the assumption that each sample shows the same level of qPCR inhibition. However, in our experience this is often not the case.
The second method, in which the internal control target *cry1* is used as correction for qPCR inhibition in each individual sample has the assumption that each target is affected by qPCR inhibition to the same extent.
Furthermore, quantification of *C. burnetii* DNA within samples is most accurately assessed using single copy genes, like *com1*. The *com1* gene is present in a single copy within the *C. burnetii* genome, and can be related to a single organism of *C. burnetii* (1 copy of target *com1* = 1 *C. burnetii* genome = 1 organism). This in contrast to the multicopy insertion element *IS1111*, which can be present in multiple copies within the *C. burnetii* genome (17).

Quantification using only this *C. burnetii* target is not very accurate. First, the number of *IS1111* copies within the genome of the *C. burnetii* strains circulating in the Netherlands is unknown. In addition, even when this last information can be obtained, an assumption has to be made that each *IS1111* copy is amplified with the same efficiency for accurate quantification.

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