



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

Q fever: the answer is blowing in the wind

*Detection of *Coxiella burnetii* in aerosols*

RIVM letter report 330291005/2011

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Arnout de Bruin (Researcher)
National Institute for Public Health and the Environment

Rozemarijn van der Plaats (Research Technician)
National Institute for Public Health and the Environment

Ingmar Janse
National Institute for Public Health and the Environment

Bart van Rotterdam (Project Leader)
National Institute for Public Health and the Environment

Contact:
Bart van Rotterdam
Laboratory for Zoonoses and Environmental Microbiology (LZO)
bart.van.rotterdam@rivm.nl

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

Abstract

Detection of *C. burnetii* DNA in aerosols

Coxiella burnetii is a bacterium that causes Q fever, a zoonosis that affects large numbers of both humans and animals. From 2007 to 2010, large outbreaks of Q fever were observed in a rural area in the Netherlands. In 2009, field studies were started to investigate if *C. burnetii* DNA can be detected in aerosols on and in the near vicinity of Q fever affected farms. In 2010, these studies were continued in two areas studied in 2009, in the provinces of Noord-Brabant and Zuid-Limburg, to investigate if *C. burnetii* DNA was still present in aerosols in these areas.

In both areas, the *C. burnetii* DNA content in aerosols obtained in 2010 seemed to have declined in comparison to data of the same locations visited in 2009. These data are in agreement with the observed reduction in the number of reported Q fever cases in 2010 in comparison to 2009. Possible explanations for this decline could be the start of a mandatory vaccination campaign for small ruminants in 2009 and the culling of pregnant animals on Q fever affected farms that started at the end of 2009.

This data will be used in future investigations, in which we will combine molecular detection and typing methods for *C. burnetii* in aerosols with mathematical modelling to get more insight in the transmission of *C. burnetii* via aerosols and track (individual) sources for *C. burnetii* infection.

Keywords:

Coxiella burnetii, Bio aerosol, real time PCR, Q fever

Trefwoorden:

Coxiella burnetii, Bio aerosol, real time PCR, Q-koorts

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1 Introduction

Q fever, a zoonosis caused by the bacterium *Coxiella burnetii*, has become an emerging public health problem in the Netherlands. Three Q fever outbreaks were reported since 2007, which increased in magnitude each year¹. Goat farms are often implicated as potential sources for human Q fever^{2, 3}, which is supported by epidemiological studies carried out during the outbreaks in the Netherlands⁴⁻⁶.

The impact of the various transmission routes of *C. burnetii*, however, is not well understood. When animals are infected, the main sources of *C. burnetii* shedding to the environment are birth materials like amnion fluid and placenta material, manure, urine and milk^{7, 8}. Transmission to humans is thought to occur primarily via contaminated aerosols, generated by infected animals or animal products^{9, 10, 12, 16}. However, available data on *C. burnetii* in aerosols is sparse.

To date, several studies have reported the presence of *C. burnetii* in environmental samples^{11-13, 24}, which were not related to large outbreaks of Q fever.

To investigate the presence of *C. burnetii* in veterinary and environmental matrices on farm premises and their role in *C. burnetii* transmission to humans, source finding investigations were initiated by several Municipal Health Services (GGD) during the large Q fever outbreaks in the Netherlands between 2007 and 2009. Vaginal swabs obtained from goats and sheep and surface area swabs (accumulated dust) obtained from stables revealed that *C. burnetii* DNA is present on most farms, which were suspected to be a source for human Q fever cases in their near vicinity^{14, 25}. It was hypothesized that abortion waves on large dairy goat farms had played a predominant role in the transmission of *C. burnetii* to humans. During delivery by Q fever positive goats and sheep, large numbers of *C. burnetii* can be dispersed into the air via the formation of aerosols¹⁵⁻¹⁸. Furthermore, during delivery, *C. burnetii* laden amniotic fluids and placenta material contaminate the layers of straw covering the stable floors of farms. When stable capacity is reached, these layers are removed from the stable and stored on the farm premises. Secondary aerosol formation in stables, e.g. during handling of manure contaminated straw can also result in the formation of so-called secondary aerosols containing *C. burnetii*.

The current study is a continuation of the study conducted in 2009¹⁹ to investigate whether aerosols, obtained during and after Q fever outbreaks, on and in the near vicinity of Q fever affected farms, contain *C. burnetii* DNA.

The goal of the present study is to get insight in possible transmission of *C. burnetii* via aerosols. This, in turn, can give us clues and generate ideas on how to set-up future investigations in which a combination of aerosol sampling, molecular typing and mathematical modelling can be used to identify (individual) sources for *C. burnetii* infection.

2 Area selection and detection of *Coxiella burnetii* DNA in aerosols

2.1 Area selection for aerosol sampling

2.1.1 Areas selected in 2009

In 2009, a study to investigate the presence of *C. burnetii* DNA in aerosols was conducted on farms suspected to be involved in the emergence of human Q fever cases in their near vicinity¹⁹. In that study, Q fever affected farms were selected based on the following criteria: (1) Reported abortion waves among goats or sheep in 2009, or (2) A positive status in *C. burnetii* source finding investigations in 2008 or 2009. Two hereby selected farms (A & B) were thought to be involved in an emerging cluster of human Q fever cases in their near vicinity. Farm A is non-dairy sheep farm, located in the south-east of the Netherlands, nearby the village of Nuenen. Farm B is a dairy goat farm, located in the south-east of the country as well, nearby the village of Voerendaal.

Farm A was suspected to be involved in the emergence of a human Q fever cluster in Nuenen in 2009. Therefore, veterinary samples (vaginal swabs) were obtained by the Food and Consumer Product Safety Authority (nVWA) on 15-05-2009 and screened for the presence of *C. burnetii* DNA using qPCR by the National Institute for Public Health and the Environment (RIVM). In that same week, eight aerosol samples were obtained on 500m and 1000m distance from the farm in all four wind directions. No aerosol samples were obtained on the farm premises. Seventeen out of 20 vaginal swabs on farm A were found to be positive, fifteen for only multicopy target *IS1111* and two samples for both the multicopy target *IS1111* and single copy target *com1*. Seven out of eight aerosol samples were found to be positive for multicopy target *IS1111* only (Figure 1). For a description of scoring procedures, see section 2.4.

Farm B, a dairy goat farm, was suspected to be involved in the emergence of a human Q fever cluster in Voerendaal in 2009. This farm reported an abortion wave in 2009 and Q fever among goats was diagnosed by the Animal Health Service (GD) using serology and PCR. In addition, Q fever among humans on the farm was diagnosed by the Municipal Health Service (GGD) Zuid-Limburg. This farm was visited three times by employees of the National Institute of Public Health and the Environment during the 2009 Q fever outbreak at weeks 14, 21 and 30. A total of four aerosol samples were collected on each visit in a radius of 1000 m distance from the farm in all four wind directions (Figure 2). In weeks 14 and 30, all four aerosol samples were found to be positive for both *C. burnetii* targets *com1* and *IS1111*. In week 21, three aerosol samples were found positive for *C. burnetii* target *IS1111* only, and one aerosol sample was scored as negative.

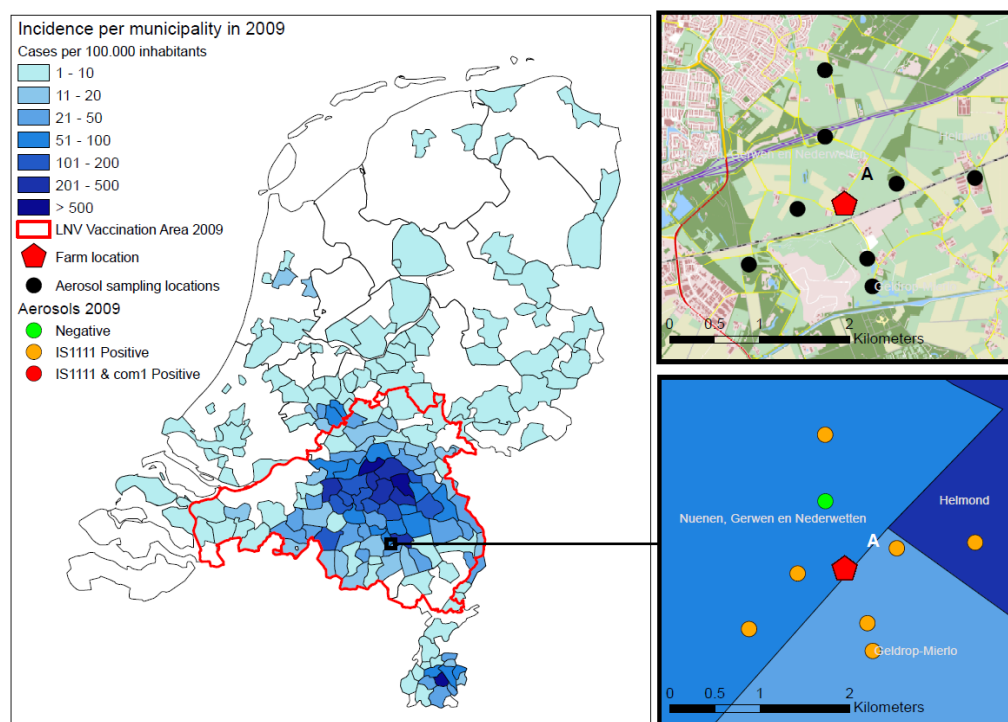


Figure 1. Screening for *C. burnetii* DNA in aerosols in the near vicinity of a Q fever affected sheep farm (A) in 2009. In the right top panel, aerosol sampling locations on 500m and 1000m distance from the farm are indicated by black circles. In the right panel below, orange circles indicate aerosol samples positive for multicopy target *IS1111* and green circles indicate negative aerosol samples.

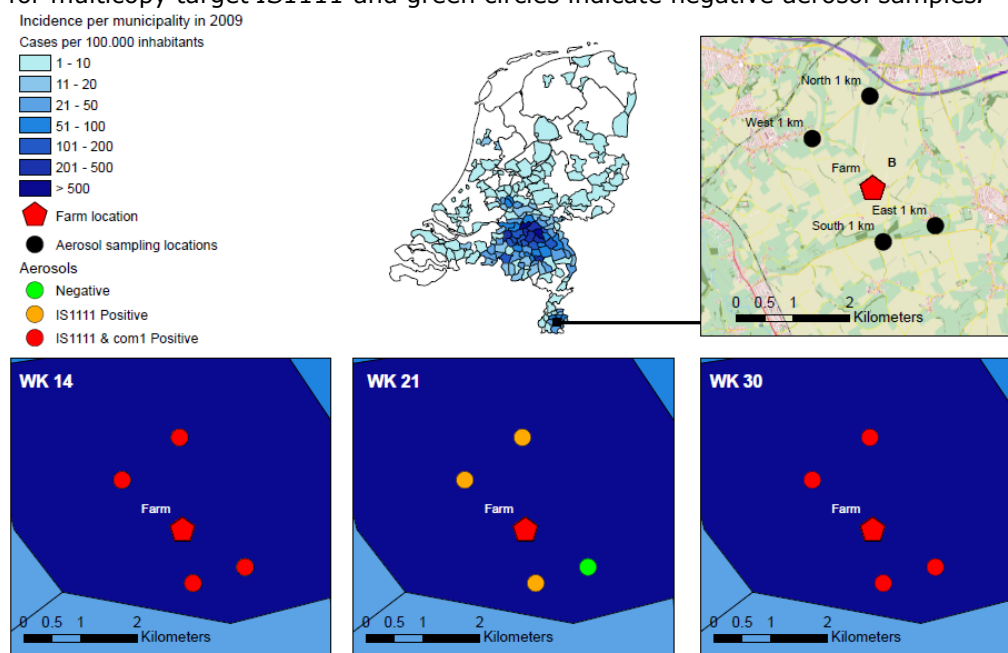


Figure 2. Longitudinal screening for *C. burnetii* DNA in aerosols in the near vicinity of a Q fever affected dairy goat farm (B) in 2009. In the right top panel, aerosol sampling locations on 1000 m distance from the farm are indicated by black circles. In the three panels below, the presence of *C. burnetii* DNA per visit is indicated by green (negative), orange (*IS1111* positive), and red (*com1* & *IS1111* positive) circles.

2.1.2 Areas selected in 2010

In 2010, the screening for *C. burnetii* DNA in aerosols was continued in both areas on a larger scale. The area visited in Nuenen, in the province of Noord-Brabant, was enlarged by selecting an area of 15 by 15 kilometres, which included the non-dairy sheep farm (A), and three Q fever positive farms screened during source finding investigation in 2009. This area was affected severally by Q fever, showing emerging clusters of human Q fever cases during the outbreaks between 2007 and 2009⁴.

For the selection of aerosol sampling locations, the 15 by 15 kilometre area was divided into nine blocks of five by five kilometres and sixteen aerosol samples were obtained from locations on the crossing point of each block, indicated by black circles in Figure 3, top right panel.

The second area is an extension of the area around farm B, located nearby the village of Voerendaal in the province of Zuid-Limburg. In 2009, four aerosol samples were obtained on a 1000 m radial distance from this farm on three successive visits (Figure 2). In 2010, the area was extended by adding a two kilometre distance radius and a total of eight aerosol samples were obtained on one and two kilometre distances from the farm in the four wind directions (Figure 3, right lower panel).

Both areas were visited in the months June, July, and September of 2010. This time period was chosen because of the dry and warm weather conditions and the emergence of a peak in human Q fever cases during the outbreaks in 2008 and 2009. The locations of the selected areas, number of aerosol samples and sampling locations can be found in Figure 3.

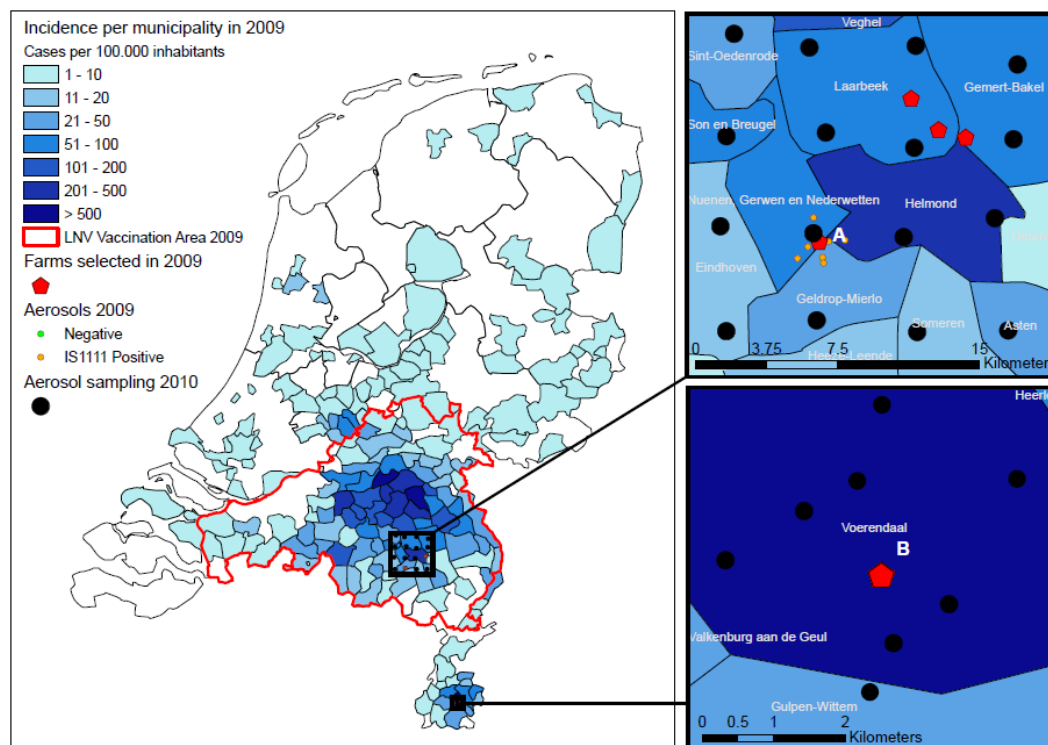


Figure 3. Aerosol sampling locations (black circles) in 2010. Both areas are an expansion of the areas visited in 2009 (see also Figures 1 and 2). The top right panel represents the area in Noord-Brabant, the right lower panel the area in Zuid-Limburg, nearby the village of Voerendaal.

2.2 Aerosol sampling

Aerosol samples were obtained using a Sartorius MD8 Airport²⁰. The apparatus was equipped with cellulose nitrate filters with a pore size of eight micrometers. Flow rate was set to 50 litres per minute with a sampling time of 10 minutes, resulting in a filtered air sample of 500 litres. Operational procedures regarding aerosol sampling and filter handling were carried out according to the manufacturer's guidelines. After sampling, filters were transferred to sterile Petri dishes, transported to the laboratory, and stored at -20°C.

2.3 Sample processing and DNA extraction

Cellulose nitrate filters were processed and DNA was extracted using the NucliSens Magnetic Extraction kit (Biomérieux, France). The cellulose nitrate filters were submerged in 10 ml of NucliSens lysisbuffer in petri dishes, which were placed on a horizontal shaker for 2 hours at 50 rpm. NucliSens lysisbuffer was transferred from the petri dishes to 15 ml Greiner tubes. As an internal process control for DNA extraction, 50 µl of a *B. thuringiensis* spore suspension (1.2×10^5 spores) was added to each sample. Samples were placed at room temperature for one hour to complete lysis. From this point onwards, DNA extraction procedures were carried out according to the manufacturer's protocol.

2.4 Detection of *C. burnetii* by multiplex real time PCR

2.4.1 Multiplex real time PCR (qPCR)

The set-up of a novel multiplex real time PCR assay (qPCR) for *C. burnetii* is described by De Bruin *et al.* (*accepted for publication*). This assay was modified to improve sensitivity and one single copy target (*icd*) was removed from the assay since one single copy target proved to be sufficient for screening purposes. For targets *com1* and *IS1111*, shorter primers and new (hydrolysis) probes were designed using software package Visual OMP 6 (Table 1). This new qPCR assay was tested in a ring trial for the detection of *C. burnetii* in veterinary samples, facilitated by the Veterinary Laboratories Agency, Weybridge, Addlestone, Surrey, United Kingdom (VLA) and results were published²¹.

For each target in the multiplex qPCR assay, probes were labelled with a different fluorophore. The probe for target *com1* was labelled with fluorophore JOE, the probe for *IS1111* with FAM, and the probe for *cry1* with fluorophore Cy5. All probes were additionally labelled with Black Hole Quencher 1. Dyes were coupled to the 5' end and quenchers to the 3' end.

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 in triplicate. Analysis of the data was performed on the software provided by Roche (Lightcycler 480 Software release 1.5.0. SP3).

2.4.2 Quantification of *C. burnetii* DNA

Due to its presence of multiple copies within the *C. burnetii* genome²², 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 isolate²², 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* & *IS1111*-positive (high *C. burnetii* DNA content). Samples were 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 used not only 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.

Primers & probe names		Primer and probe sequences (5'-> 3')	Product length
target <i>com1</i>			
forward primer	scompri_f	AGCAGCCGCTAAACAAGGAAAAT	74
reverse primer	scompri_r	GTTCTGATAATTGGCCGTCGACAC	
probe (JOE)	Tqpro_scom	ATGCTTTCCACGACGCGCTGCTC	
target <i>IS1111</i>			
forward primer	sIS1pri_f	CGGGTTAAGCGTGCTCAGTAT	75
reverse primer	sIS1pri_r	TCCACACGCTTCCATCACCAC	
probe (FAM)	Tqpro_sIS1	AGCCACCTTAAGACTGGCTACGGTGGAT	
target <i>Cry1</i>			
forward primer	sBtpri_f	AGTTCGTGTCTGTCCGGGTC	85
reverse primer	sBtpri_r	CATGAATGGTTACGCAACCTTCT	
probe (Cy5)	Tqpro_sBt	ATCCCTCCTTGTACGCTGTGACACGAAGGA	

Table 1. Primers and probes for the *C. burnetii* multiplex qPCR assay.

3 Screening for *C. burnetii* DNA in aerosols in 2010

The area in Noord-Brabant (area of farm A) was visited in weeks 22, 29, and 38 of 2010 (on 02-06, 20-07, and 22-09 respectively). On each visit 16 aerosol samples were collected and screened for the presence of *C. burnetii* DNA by qPCR. *Coxiella burnetii* DNA content in aerosol samples was found to be low, with positive samples only in the *IS1111*-positive category. In week 22, four aerosol samples were found positive, in week 29 six samples were found positive, and in week 38 four samples were found positive for *C. burnetii* target *IS1111* only (Table 2 and Figure 4). In total, 14 out of 48 aerosol samples were positive for *C. burnetii* DNA. Aerosol samples obtained from location H03 showed positive results on all three visits. Aerosol samples obtained from within 500 m distance from the farm visited in 2009 (H10 in Figure 4) showed no positive results in any of the three visits. In aerosol samples about 5 km south of the farm *C. burnetii* DNA was present during two visits: in weeks 22 and 29. In addition, during the visit in week 29, all aerosol samples on 5 km east, west, north and south of this farm were found positive for *C. burnetii* DNA. Finally, in the vicinity of the group of three Q fever positive farms, selected in source finding investigations in 2009, a number of aerosol samples were found to be positive to the north, south and east of this group of farms, especially on visits in weeks 29 and 38 (Figure 4).

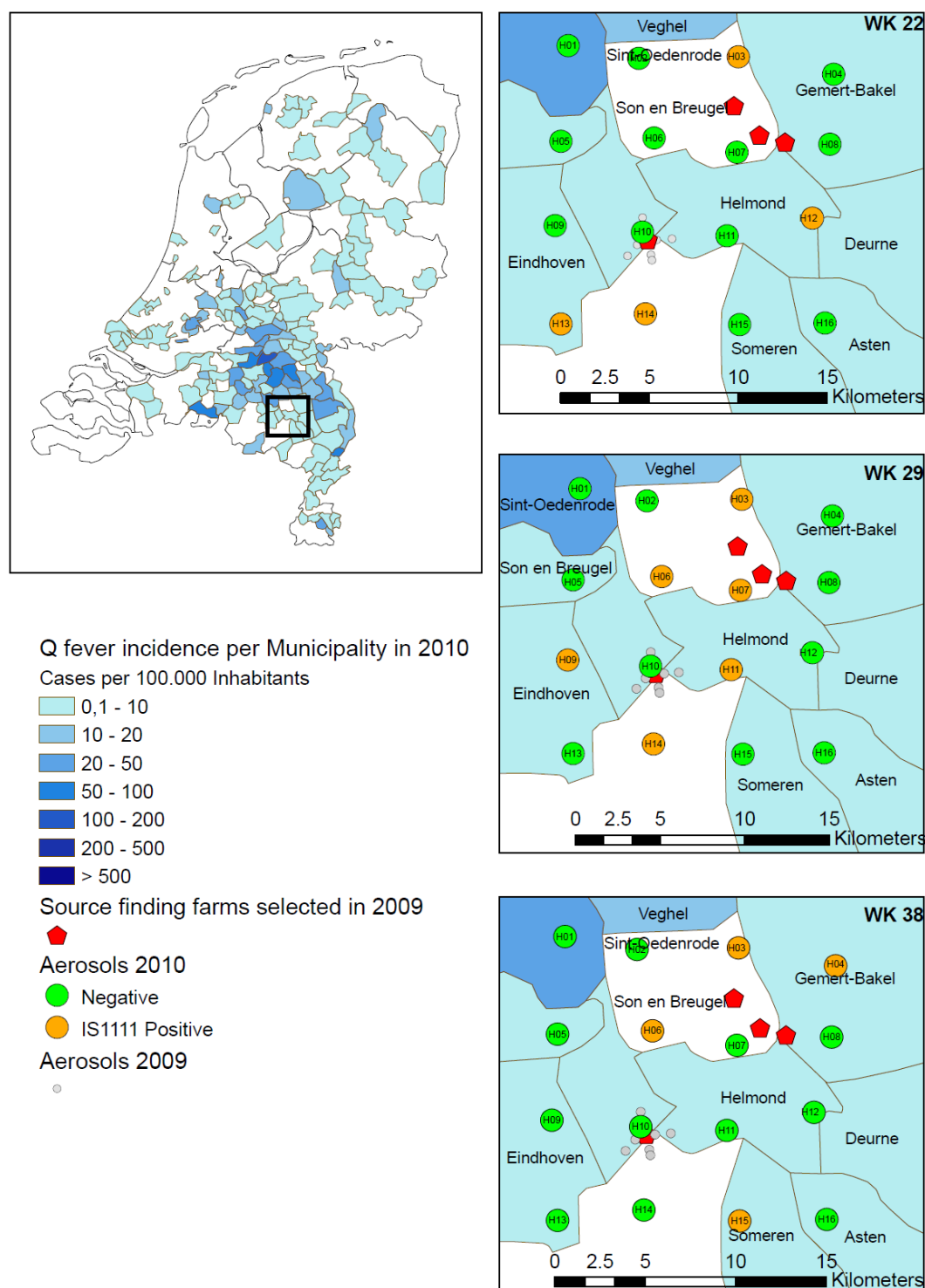
The area in Zuid-Limburg, around the selected farm in Voerendaal (farm B), was visited in weeks 22, 29, and 38 of 2010 (on 04-06, 21-07, and 21-09 respectively). On each visit eight aerosol samples were collected and screened for the presence of *C. burnetii* DNA by qPCR. *C. burnetii* DNA content in aerosols in this area was found to be low as well, with positive signals only for target *IS1111*. In weeks 22, four aerosol samples were found positive, in week 29 two aerosol samples were found positive, and in week 38 four samples were found positive for *C. burnetii* DNA (Table 2 and Figure. 5). In total, 10 out of 24 aerosol samples were positive for *C. burnetii* target *IS1111*.

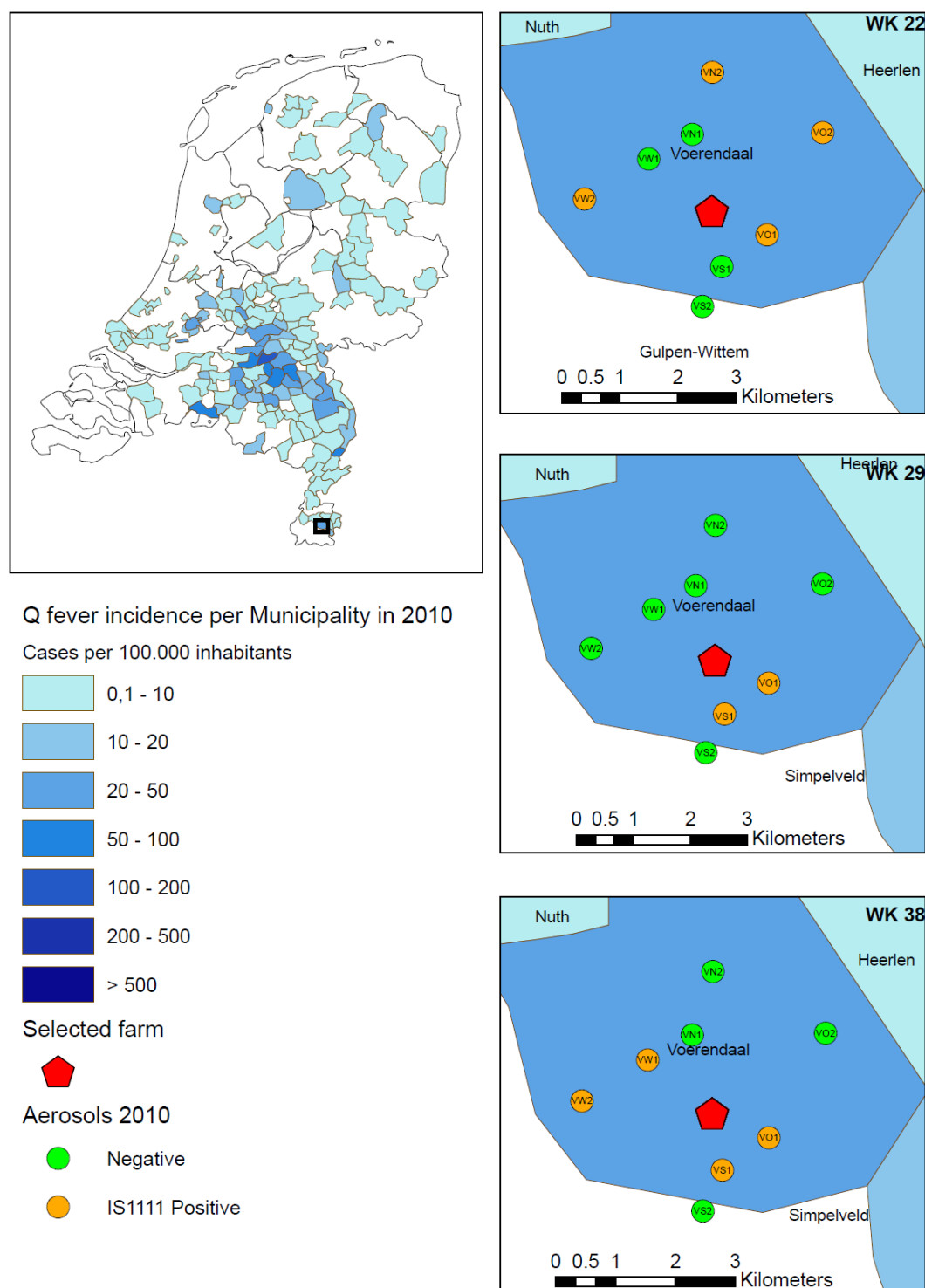
The level of *C. burnetii* DNA in aerosols collected reached the detection limit for target *IS1111*, the most sensitive target of the qPCR, and none of the aerosol samples produced positive results for single copy target *com1*. Cq values for target *IS1111* in both areas ranged between 36 and 38 in 2010, which is near the detection limit of the qPCR assay and compares to <10 copies *IS1111* per 500L of sampled air. In 2009, aerosol samples obtained from the same location within a 1000 m distance of the goat farm (B) in Voerendaal showed Cq values for target *IS1111* between 33 and 35, which can be compared to 10¹-10² copies *IS1111* per 500L of sampled air. Aerosol samples obtained in Nuenen in 2009 within 1000 m distance of the sheep farm (see Figure 1) showed Cq values for target *IS1111* between 35 and 38, which are in the range of the results found in 2010.

The single copy gene *com1* is present in one copy per *C. burnetii* genome (1 genome = 1 *C. burnetii* organism). The number of copies of target *IS1111* within the genomes of the *C. burnetii* strains circulating in the Netherlands is still unknown. Since positive results were found for the multicopy target *IS1111* only, accurate quantification of the number of *C. burnetii* organisms cannot be obtained.

Area location	Municipality of sampling location	Map label	Weeks visited in 2010		
			22	29	38
Noord-Brabant	St.-Oedenrode	H01	-	-	-
Noord-Brabant	Laarbeek	H02	-	-	-
Noord-Brabant	Laarbeek	H03	+	+	+
Noord-Brabant	Gemert-Bakel	H04	-	-	+
Noord-Brabant	Son en Breugel	H05	-	-	-
Noord-Brabant	Laarbeek	H06	-	+	+
Noord-Brabant	Helmond	H07	-	+	-
Noord-Brabant	Gemert-Bakel	H08	-	-	-
Noord-Brabant	Eindhoven	H09	-	+	-
Noord-Brabant	Nuenen, Gerwen & Nederwetten	H10	-	-	-
Noord-Brabant	Helmond	H11	-	+	-
Noord-Brabant	Helmond	H12	+	-	-
Noord-Brabant	Eindhoven	H13	+	-	-
Noord-Brabant	Geldrop-Mierlo	H14	+	+	-
Noord-Brabant	Someren	H15	-	-	+
Noord-Brabant	Asten	H16	-	-	-
Zuid-Limburg	Voerendaal	VN1	-	-	-
Zuid-Limburg	Voerendaal	VN2	+	-	-
Zuid-Limburg	Voerendaal	VO1	+	+	+
Zuid-Limburg	Voerendaal	VO2	+	-	-
Zuid-Limburg	Voerendaal	VS1	-	+	+
Zuid-Limburg	Voerendaal	VS2	-	-	-
Zuid-Limburg	Voerendaal	VW1	-	-	+
Zuid-Limburg	Voerendaal	VW2	+	-	+

Table 2. Screening for *C. burnetii* DNA in aerosols in areas Noord-Brabant and Zuid-Limburg in 2010. *IS1111*-positive samples are indicated by symbol +, negative samples by symbol -.





4 Discussion

Since it was hypothesized that dairy goat farms play an important role in the past Q fever epidemic in the Netherlands, the Food and Consumer Product Safety Authority (nVWA) initiated control measures, which are constantly updated¹. The positive outcome of bulk milk screening, carried out by the nVWA in close collaboration with the Animal Health Service (GD) and the Central Veterinary Institute (CVI), led to culling of all pregnant dairy goats on the farm in Voerendaal in Zuid-Limburg in December of 2009. In March of 2010, the farm changed its business operations from a dairy goat farm to a veal calves production farm. The quantity of *C. burnetii* DNA found in aerosols in 2010, based on Cq values for targets *IS1111* & *com1*, was lower than in 2009 in this area. This is in agreement with a reduced number of human Q fever cases in the same area in 2010 in comparison to 2009.

In 2009, all aerosol samples but one in the vicinity of the sheep farm in Nuenen (farm A) were positive for *C. burnetii*. In 2010, aerosols obtained from a comparable distance from this farm (within 500 m: H10 in Figure 4) showed no positive results in any of the three visits. The farm is still operational as a non-dairy sheep farm and the sheep has been vaccinated twice before aerosol sampling started in June of 2010.

These findings are in agreement with a reduced number of human Q fever cases in the same area in 2010 in comparison to 2009. Possible explanations for this decline could be the start of a mandatory vaccination campaign for small ruminants in 2010 and the culling of pregnant animals on Q fever affected farms.

Since studies on aerosol screening for *C. burnetii* started in 2009, no information on background levels of *C. burnetii* in aerosols in these areas is available from before that time period. Therefore, it is not clear if the reduction of *C. burnetii* in aerosols from 2009 to the present level in 2010 has reached the level of normal background levels for *C. burnetii* in the environment before the outbreaks. In a recent study it was shown that *C. burnetii* can be detected in aerosols, without a recent outbreak of Q fever²⁴. The *C. burnetii* content in that study, however, cannot be easily compared to our studies. Although screening aerosols for *C. burnetii* presence is still experimental, we have shown that *C. burnetii* contaminated aerosols are present in the air in a Q fever affected area.

This data will be used in future investigations, in which we will combine molecular detection and typing of *C. burnetii* in aerosols with mathematical modelling to get more insight in the transmission of *C. burnetii* via aerosols and track (individual) sources for *C. burnetii* infection. Therefore, we have to optimize aerosol sampling procedures, DNA extraction methods, and procedures to account for possible qPCR inhibition to be able to quantify the number of *C. burnetii* organisms in these matrices

¹ Dutch Ministry of Economic Affairs, Agriculture and Innovation: Factsheet maatregelen Q-koorts (14 september 2010).

5 Conclusions

- In 2010, *C. burnetii* DNA is still found in aerosols obtained within two km distance of a dairy goat farm in the area of Zuid-Limburg that was affected by Q fever in 2009.
- The quantity of *C. burnetii* DNA in aerosols, based on the number of samples with positive signals for targets *IS1111* & *com1*, measured on the same locations in Zuid-Limburg was lower in 2010 compared to 2009.
- A number of aerosol samples in the vicinity of a group of dairy goat farms in the area of Noord-Brabant, selected for screening during source finding investigations in 2009, were found positive during all three visits in 2010.
- In general, a reduction in the number of *C. burnetii* positive aerosol samples corresponds to a lower human incidence of Q fever, as well as the presence of vaccinated sheep versus unvaccinated sheep for the selected area in Noord-Brabant and to the absence of goats in the Voerendaal region respectively.

6 Literature

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