



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

## **Molecular typing of *Coxiella burnetii* from source finding samples taken in 2009**

RIVM letter report 330291006/2011  
A. de Bruin



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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 Zoonosen

## Abstract

### **Molecular typing of *Coxiella burnetii* from source finding samples taken in 2009**

Q fever, caused by the bacterium *C. burnetii*, is a zoonosis with a worldwide distribution that affects both humans and animals. From 2007 to 2010, large community outbreaks of Q fever were observed in the Netherlands.

In 2008 and 2009 source finding investigations were initiated by several Municipal Health Services, primarily on commercial dairy goat farms, to pinpoint potential sources for Q fever. In that same year, the Food and Consumer Product Safety Authority initiated a project to investigate if petting zoos were a potential source for human Q fever as well.

Petting zoos showed insufficient *C. burnetii* DNA content for molecular typing (qPCR Cq values higher than 33) and were not considered an important source for human Q fever. However, 31 samples from eight out of 57 commercial dairy farms, involved in source finding investigations in 2009, showed a relatively high *C. burnetii* DNA content based on qPCR data for single copy target *com1* (Cq values lower than 33). These samples were selected for molecular typing using a Multi-Locus Variable number of tandem repeat Analysis (MLVA).

In this study we show that samples highly positive for *C. burnetii* DNA can be successfully typed using a multiplex MLVA assay. Three different MLVA types were found, based on six MLVA markers. On seven out of eight locations a single MLVA type was found. On one location a mixture of two types was observed within a number of samples.

Our findings show that multiple MLVA types are present in the Netherlands, which is promising for future source finding investigations to identify potential sources. However, only a few different MLVA types have been determined in human and animal samples so far, which makes identifying transmission routes and sources of *C. burnetii* in the Netherlands still challenging.

Keywords:

*Coxiella burnetii*, Molecular typing, MLVA, Q fever, Source finding

Trefwoorden:

*Coxiella burnetii*, Moleculaire typering, MLVA, Q-koorts, Bronopsporing



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## 1 Molecular typing of *Coxiella burnetii*

### 1.1 Molecular typing methods for *C. burnetii*

Distribution patterns of pathogen strains are very important in source finding investigations, identifying transmission routes and epidemiological studies. Genes or regions within the genome selected for molecular typing should be variable in order to be able to differentiate between strains circulating in animal and/or human population and the environment.

Over the years, a number of different molecular typing methods have been developed for *C. burnetii*. One of the first molecular typing methods for *C. burnetii* is based on restriction fragment length polymorphism (RFLP) in combination with pulse field gel electrophoresis (PFGE)<sup>1, 2</sup>. More recently, PCR based methods were developed for molecular typing of *C. burnetii* strains, like multi-locus variable number of tandem repeats analysis (MLVA)<sup>3, 4</sup>, multi-locus sequence typing (MST)<sup>5</sup>, and Single Nucleotide Polymorphism (SNP)<sup>6</sup>.

The current report describes the findings of molecular typing of *C. burnetii* DNA using MLVA, obtained from samples taken during source finding investigations on suspected Q fever affected farms in 2009. Typing of *C. burnetii* DNA collected in the Netherlands during source finding investigations in 2008 is described in RIVM Report 330291002<sup>7</sup>.

### 1.2 Multi-Locus Variable number of tandem repeats Analysis (MLVA)

The PCR based molecular typing technique MLVA is a method to sub-type microbial isolates based on the Variable copy Numbers of Tandem Repeats (VNTR) at several loci. A VNTR typically exhibits a large range of copy numbers, even among highly related bacterial species. For a selected set of tandem repeats, comparison of the number of copy numbers between bacterial strains can be used to obtain insight in relationships at a micro-evolutionary level.

*Coxiella burnetii* strains may differ in the number of repeats within a specific locus (VNTR), and a number of different loci can be combined to obtain specific MLVA types. One often used MLVA for *C. burnetii* has been developed at RIVM<sup>4</sup>, in which 16 *C. burnetii* isolates and five passage history/laboratory variants were characterized using seven loci. Another more extensive MLVA assay<sup>3</sup> was developed in France using 17 loci, including the seven loci used by RIVM. More recently, a multiplex MLVA PCR assay was developed<sup>8</sup>, which includes six loci from the assay developed in France.

As molecular typing requires a significant amount of relatively uncontaminated DNA (free from inhibitors), MLVA was only attempted in our studies on DNA extracts with relatively high *C. burnetii* DNA content. Samples were selected from a large pool of DNA extracts, obtained from animals from 97 petting zoos (1382 samples) and 57 commercial dairy farms (922 samples) in 2009.

DNA extracts showing Cq values of 33 or lower for *C. burnetii* target *com1* in qPCR assays, were selected for molecular typing. When samples with higher Cq values were used (lower *C. burnetii* DNA content) molecular typing was not successful. A Cq value of 33 for single copy target gene *com1* correspond roughly to an amount between 10<sup>2</sup>-10<sup>3</sup> *com1* copies per vaginal swab, which translates to the same amount of *C. burnetii* organisms, because target *com1* is present in single copy within a *C. burnetii* genome.

### 1.3 Design of a multiplex MLVA PCR assay for *C. burnetii*

MLVA typing of samples using the methods described by RIVM<sup>4</sup> and in France<sup>3</sup> is relatively time consuming, requires large amounts of DNA template, and is costly in terms of consumables. In 2009 we were able to participate in the development of a multiplex MLVA assay for *C. burnetii*<sup>8</sup>. In this multiplex assay, five MLVA markers developed by RIVM and one MLVA marker developed in France were incorporated into two separate multiplex PCR assays, each including three markers. Despite extensive efforts, we were not able to obtain reproducible results using these PCR assays. The PCR reactions produced no amplified template at all for any of the samples collected during source finding investigations in 2009.

We therefore modified the primer sets for one of the multiplex PCRs, and developed a completely new one for three MLVA loci. Details of both PCR assays can be found in Table 1. The two multiplex MLVA PCR assays were tested on DNA of three *C. burnetii* strains, or isolates: *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*) (data not shown). MLVA PCR-assays were carried out on a PCR-express thermal-cycler (Thermo-Scientific, Breda, the Netherlands). The separation of PCR fragments was performed on an ABI 3700 DNA sequencer (Applied Biosystems, Foster City, CA) using the standard GeneScan module. The GeneScan data were imported into Genemarker and the Bionumerics 4.0 software package (Applied Maths) for analysis.

Table 1: Primer sets for typing of 6 loci in 2 multiplex MLVA PCR assays. Primer sequences, repeat sequences and repeat length are given for the 6 selected *C. burnetii* loci.

Marker*	Primer name	Primer sequence (5'-3')	Repeat sequence	Repeat length (bp)
<i>Multiplex assay 1</i>				
Ms34 (Cox1)	Forward primer	ATCAGCGACTCGAAGAAAA	GAAAAG	6
	Reverse primer	AGGGTGACTTTTCACTTAAAG		
Ms27 (Cox2)	Forward primer	CGCTATTTTTTCAGTTTGAGTAA	TGAAGA	6
	Reverse primer	@TCGCAAACGTGCGACT		
Ms28 (Cox5)	Forward primer	CGATGACGACAAAAAGACT	TAAGAA	6
	Reverse primer	@CGGTAATTACTGTAAATAAATACAAAGACA		
<i>Multiplex assay 2</i>				
Ms24 (Cox4)	Forward primer	CCATTGTGTAATTGACATGAAGAA	GACGGAA	7
	Reverse primer	GCCACACAACCTGTGTTTCAG		
Ms31 (Cox7)	Forward primer	CCGGTATTCTAACCAACTGAAC	CAGAGGA	7
	Reverse primer	GAATCCCTCAGACCCATTTC		
Ms20	Forward primer	TGTAACAGCACCGCCTGA	GGAAGAAGCGCCACCCG AAATAGGGTTGCCTCC	33
	Reverse primer	@CTTGCCCTTCTTGATTTTC		

\*Marker name = Arricau-Bouvery *et al.* 2006 (Svraka *et al.* 2006)

@ = non-matching

## 2 MLVA typing of *C. burnetii* during source finding investigations

### 2.1 Selection of samples and molecular typing of *C. burnetii*

During 2009, Q fever source finding investigations were carried out on 57 selected commercial dairy farms. On these farms, veterinary and environmental samples were collected and screened for the presence of *C. burnetii* DNA using qPCR<sup>9</sup>. In addition, potential animal sources were sampled on 97 petting zoos and screened for *C. burnetii* DNA as well<sup>10</sup>.

The level of *C. burnetii* DNA isolated from the samples taken on the petting zoos turned out to be insufficient for molecular typing without prior cultivation. Using this knowledge we switched to samples collected at several farms for source finding investigations as they generally contained higher amounts of *C. burnetii* DNA. To get insight in the number of strains circulating on commercial dairy goat or sheep farms in the Netherlands, eight farms were selected that showed the highest *C. burnetii* DNA content. In total, 31 DNA extracts obtained from *C. burnetii* positive vaginal swab samples were selected for MLVA typing. Samples selected showed Cq values <33 for target *com1*, used in the qPCR detection assays during source finding investigations in 2009.

MLVA typing of the 31 animal samples using the six MLVA markers revealed that the number of variant alleles per locus varied between one and three. Based on these six markers, three different MLVA types, denoted as type A, B and C, were detected amongst the 31 analysed samples. MLVA type A was found at seven of the eight selected locations. *C. burnetii* type B was found on one location only and was represented by only one sample. *C. burnetii* type C was found on one single location as well and was found both combined with type A in eight samples, and as the single type in one sample on this location. (Table 2 and Figure 1). In Figure 1, the size of the pie-chart is proportional to the number of samples in that location. The number in the pie-charts corresponds to the farm number in Table 2. The colours green, red and blue indicate the different MLVA types (A, B, and C respectively). On one location, two MLVA types (A & C) were detected simultaneously in seven out of nine samples, this is indicated by the colour purple.

### 2.2 Confirmation sequencing of the number of tandem repeats per marker

All obtained PCR fragments for each marker and the number of containing tandem repeats were confirmed by sequencing. A small discrepancy between sequencing results and PCR fragment analyses by capillary electrophoresis was found for marker MS34. When the numbers of tandem repeats within PCR products for this marker are calculated based on fragment analysis by mass, one repeat less is obtained for all four measured fragments in comparison to the sequencing results for this marker (Table 3). This was accounted for by comparing PCR product lengths obtained by fragment analysis with the PCR product length obtained from sequence information for each marker.

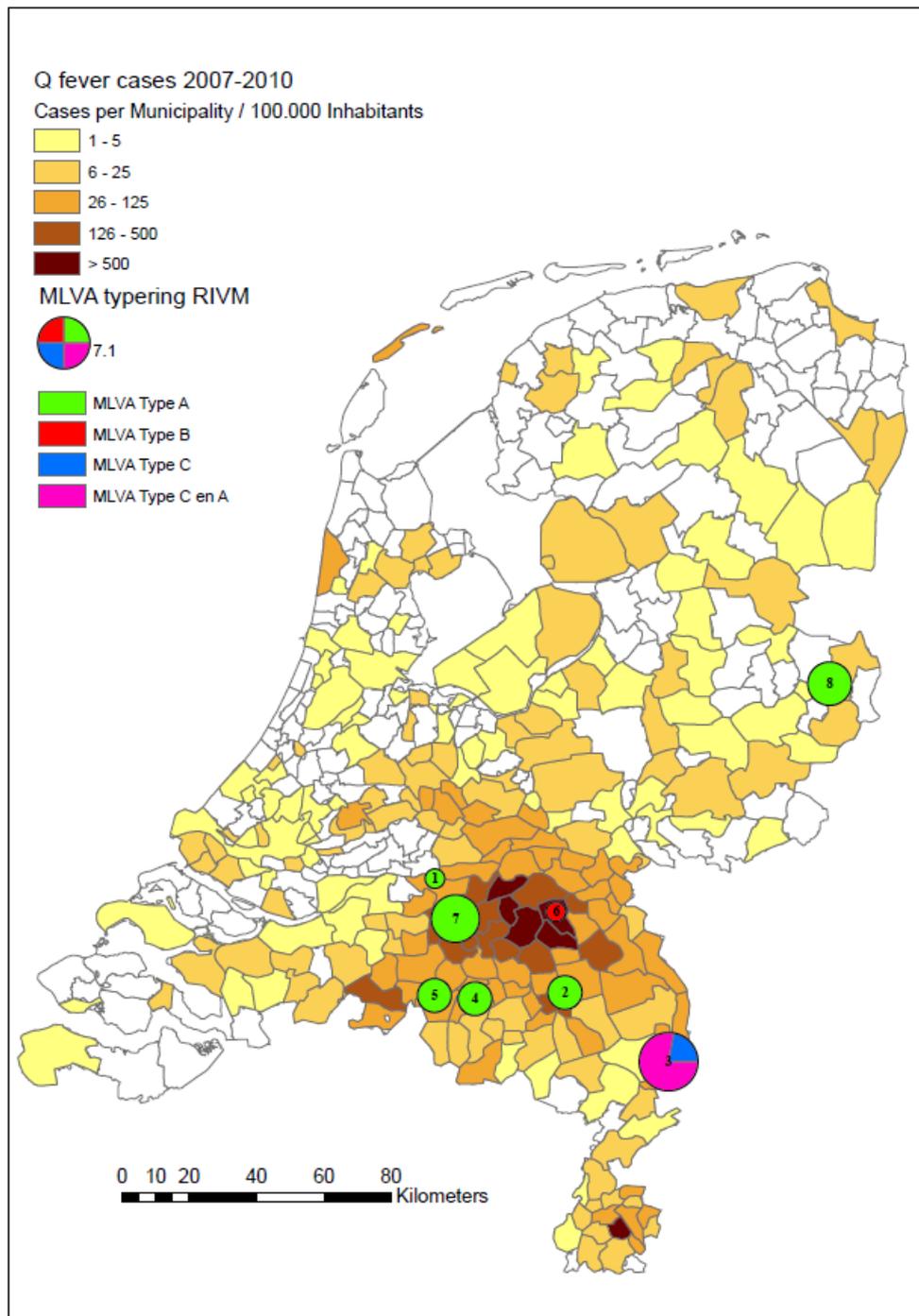
Farm number	Location	Sample number	MLVA Type	MLVA Loci					
				Ms34	Ms28	Ms27	Ms31	Ms20	Ms24
1	Aalst	1	A	7	3	3	1	7.5	10
		1	A	7	3	3	1	7.5	10
		2	A	7	3	3	1	7.5	10
2	Bakel	2	A	7	3	3	1	7.5	10
		3	A	7	3	3	1	7.5	10
		3	A	7	3	3	1	7.5	10
3	Belfeld	1	A + C	2 & 7	5	4	1	4.5	8
		2	A + C	2 & 7	5	4	1	4.5	8
		3	A + C	2 & 7	5	4	1	4.5	8
		4	C	2	5	4	1	4.5	8
		5	A + C	2 & 7	5	4	1	4.5	8
		6	A + C	2	3 & 5	4	1	4.5	8 & 10
		7	A + C	2 & 7	3 & 5	4	1	4.5	8 & 10
		8	A + C	2	5	4	1	4.5	8 & 10
		9	A + C	2	3 & 5	4	1	4.5	8
4	Haghorst	1	A	7	3	3	1	7.5	10
		2	A	7	3	3	1	n.d.	10
		3	A	7	3	3	1	7.5	10
5	Hilvarenbeek	1	A	7	3	3	1	7.5	10
		2	A	7	3	3	1	7.5	10
		3	A	7	3	3	1	7.5	10
6	Schaijk	1	B	8	3	3	1	7.5	10
		1	A	7	3	3	1	7.5	10
		2	A	7	3	3	1	7.5	10
7	Vlijmen	3	A	7	3	3	1	7.5	10
		4	A	7	3	3	1	7.5	10
		5	A	7	3	3	1	7.5	10
		6	A	7	3	3	1	7.5	10
		6	A	7	3	3	1	7.5	10
8	Weerselo	1	A	7	3	3	1	7.5	10
		2	A	7	3	3	1	7.5	10
		3	A	7	3	3	1	7.5	10
		4	A	7	3	3	1	7.5	10
		5	A	7	3	3	1	7.5	10

Table 2. MLVA-typing of 31 DNA extracts obtained from positive vaginal swab samples from 8 different locations in the Netherlands.

MLVA locus	Label	Fragments	PCR product length (bp)		Number of tandem repeats	
			Fragment analysis	Sequencing	Fragment analysis	Sequencing
Ms34	FAM	1	107	112	1.2	2,0
		2	136.3	142	6.1	7,0
		3	142.8	148	7.1	8,0
Ms27	NED	1	290,0	288	3.3	3,0
		2	295.4	294	4.2	4,0
Ms28	VIC	1	189	190	2.8	3,0
		2	200.8	202	4.8	5,0
Ms24	NED	1	165.2	163	8.3	8,0
		2	178.6	177	10.2	10,0
Ms31	FAM	2	130.1	n.d.	1.2	n.d
Ms20	VIC	2	269.1	273	4.9	5,0
		3	348.5	354	7.3	7.5

Table 3. Sequence confirmation of the number of repeats within PCR products for each marker. n.d. = not determined

Figure 1. *C. burnetii* MLVA types found on 8 farms involved in source finding investigations in the Netherlands in 2009.





### 3 Discussion

Only 14 out of the 97 petting zoos screened for presence of *C. burnetii* in 2009 showed one or more positive samples<sup>10</sup>. The quantity of *C. burnetii* DNA, based on the absence of Cq values for target *com1* was found insufficient for successful molecular typing using MLVA. To gain insight in the number of *C. burnetii* strains circulating in the Netherlands, 31 clearly positive samples with high *C. burnetii* DNA content obtained from eight farms involved in source finding investigations in 2009 were selected for molecular typing using MLVA.

In RIVM Report 330291002<sup>7</sup>, four MLVA types (A-D) were found during source finding investigations in 2008. In that report, it was concluded that a large number of markers failed to reproduce PCR products of sufficient quantity and quality, due to inhibiting substances or low yield of DNA in these samples. Therefore, sequence efforts for confirmation of the number of repeats within the PCR products were not successful. Two samples obtained from the location in Groesbeek in 2008 were retyped successfully again using the newly developed multiplex MLVA assays. These samples were again categorised as MLVA type A. Due to the high amount of DNA template material needed for the MLVA PCR assay used in the RIVM report 330291002<sup>7</sup>, it is not possible to type all other samples used in that study.

The most commonly used molecular typing method for *C. burnetii* in the Netherlands is by MLVA<sup>11, 12</sup>. Estimations of the number of *C. burnetii* strains in patients<sup>12</sup>, dairy livestock<sup>11</sup>, and this study, showed that a single *C. burnetii* MLVA type is dominant in the Netherlands. This makes pinpointing individual sources for human Q fever more difficult because the dominant *C. burnetii* type (A) may be present on several farms and patients simultaneously. A comparison between MLVA types found in clinical samples<sup>12</sup>, and veterinary samples in this study showed that the most common MLVA types were found in both humans and animals. MLVA typing was applied on serum samples of Q fever patients<sup>12</sup> and the most common genotype observed was a genotype named MLVA type G. The authors use two multiplex MLVA PCR assays of six markers in total and four of these markers can be compared to our multiplex MLVA assay (MS24, MS27, MS28, and MS34). MLVA type G observed in human serum samples showed a close similarity with our most common genotype: MLVA type A found in veterinary samples. The number of repeats for markers MS27, MS28, and MS34 were identical for the two different MLVA methods.

However, a direct comparison of MLVA types between laboratories remains difficult because of the different MLVA assays used, and the lack of consensus in scoring the number of tandem repeats for each marker<sup>13</sup>. Even when the different MLVA assays target the same markers, minor alterations in primer binding sites can alter the scoring of alleles and therefore the outcome of the analyses. For instance, the most common genotypes observed in serum<sup>12</sup> and this study (MLVA type G and MLVA type A respectively) showed a difference of one repeat only for Marker MS24. This may be explained by differences in scoring, which in MLVA is primarily based on fragment analysis.



## 4 Conclusions

The amount of *C. burnetii* DNA found in samples from petting zoos in 2009 was insufficient for molecular typing using MLVA (Cq values above 33). We used clearly positive samples from 2009 source finding investigations instead (Cq values below 33).

Molecular typing of *C. burnetii* was successful on 31 samples having Cq values below 33 for target *com1* in the qPCR detection assay for *C. burnetii*.

Molecular typing of 31 samples obtained from eight farms involved in source finding investigations in 2009 revealed three distinct MLVA types, with one clearly dominant type.



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