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Genotypic Diversity of *Coxiella burnetii* in the 2007-2010 Q Fever Outbreak Episodes in The Netherlands

Jeroen J. H. C. Tilburg,[a] John W. A. Rossen,[b] Erik J. van Hannen,[c] Willem J. G. Melchers,[d] Mirjam H. A. Hermans,[e] Jeroen van de Bovenkamp,[f] Hendrik Jan I. J. Roest,[g] Arnout de Bruin,[h] Marrigje H. Nabuurs-Franssen,[a,d] Alphons M. Horrevorts,[a] and Corné H. W. Klaassen[a]

Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands[a]; Laboratory of Medical Microbiology and Immunology, St. Elisabeth Hospital, Tilburg, The Netherlands[b]; Laboratory of Medical Microbiology and Immunology, St. Antonius Hospital, Nieuwegein, The Netherlands[c]; Department of Medical Microbiology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands[d]; Molecular Diagnostics, Jeroen Bosch Hospital, 's-Hertogenbosch, The Netherlands[e]; Laboratory of Medical Microbiology, Stichting PAMM, Veldhoven, The Netherlands[f]; Department of Bacteriology and TSE's, Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands[g]; and Laboratory for Zoonoses and Environmental Microbiology, National Institute for Public Health and the Environment, Bilthoven, The Netherlands[h]

The genotypic diversity of *Coxiella burnetii* in clinical samples obtained from the Dutch Q fever outbreak episodes of 2007-2010 was determined by using a 6-locus variable-number tandem repeat analysis panel. The results are consistent with the introduction of one founder genotype that is gradually diversifying over time while spreading throughout The Netherlands.

From 2007 to 2010, The Netherlands was confronted with a large and unprecedented Q fever outbreak, with thousands of affected individuals (4). The increase in human cases coincided with an increase in abortions among goats (2, 4, 6). Genotypic characterization of the involved isolates can give fundamental insight into the epidemiology of Q fever in The Netherlands, allowing, for example, spread of the involved genotype(s) throughout The Netherlands during the subsequent outbreak years and/or displaying a correlation between human and animal Q fever cases. Recently, genotyping by using a 10-locus multiple-locus variable-number tandem repeat analysis (MLVA) panel revealed one predominant genotype among goats and sheep throughout the affected area (5). A 3-locus MLVA panel performed directly on clinical samples from a minor part of the affected region showed that Dutch farm animals and patients appeared to be infected by different but closely related MLVA genotypes (3). In this study, we determined the temporal and spatial diversity of *Coxiella burnetii* genotypes in human samples collected during the 2007-2010 Q fever outbreak episodes from the entire affected part of The Netherlands using a 6-locus MLVA panel.

The presence of *C. burnetii* DNA in a variety of clinical samples was determined using a real-time PCR targeting the IS*1111a* insertion element of *C. burnetii* as described earlier (8). We determined the MLVA genotype using 3 hexanucleotide repeat markers (Ms27, Ms28, and Ms34) and 3 heptanucleotide repeat markers (Ms23, Ms24, and Ms33) (1) directly in 46 Q fever-positive clinical specimens collected from acute and chronic Q fever patients. These samples were collected during the 2007-2010 outbreak episodes (Table 1 and Fig. 1A). A multicolor multiplex format was chosen to make more efficient use of the small amounts of *C. burnetii* DNA generally obtained from clinical samples. The MLVA primers for markers Ms27, Ms28, and Ms34 have been described before (3). MLVA primers were 5′-HEX-CGCMTAGCGACACAACCAC-3′ and 5′-GACG GGCTAAATTACACCTGCT-3′ for Ms23, 5′-FAM-TGGAGGG ACTCCGATTAAAA-3′ and 5′-GCCACACAACTCTGTTTTCA G-3′ for Ms24, and 5′-TAMRA-TCGCGTAGCGACACAACC-3′ and 5′-GTAGCCCGTATGACGCGAAC-3′ for Ms33, where HEX is hexachlorofluorescein, FAM is 6-carboxyfluorescein, and TAMRA is 6-carboxytetramethylrhodamine.

Multiple different but apparently closely related MLVA genotypes, A to H, were identified in 33 clinical samples covering both acute Q fever patients (e.g., sputa, bronchoalveolar lavage [BAL] fluid, throat swabs) as well as chronic Q fever patients (e.g., heart valves, aorta tissue) (Table 1). A partial MLVA genotype (assigned as "p") was obtained from another 13 samples that contained insufficient DNA to obtain a full profile. In all but one of the clinical samples that yielded a partial genotype, the same alleles were identified as those found in samples yielding a full genotype (Table 1). Clustering of the MLVA genotypes using the minimum spanning tree method showed a high degree of genetic similarity between the Dutch MLVA genotypes (Fig. 1B). Specifically, all but one of the obtained Dutch MLVA genotypes are interconnected by repeat number changes in one of the six markers (this involved either Ms23, Ms24, Ms27, and Ms34). One sample (Q056) yielded a genotype that differed in two markers from the other genotypes, and the alleles that were found in these two markers were also different from those observed in the other Dutch samples (Table 1). In contrast, the genotypes from five sequenced *C. burnetii* strains all differed in at least 3 markers from the Dutch genotypes. Negative control samples neither yielded a positive PCR result nor an MLVA result. The geographical distribution of the MLVA genotypes is shown in Fig. 1A. From the two genotypes that were observed most frequently (i.e., genotypes A and G), the G genotype apparently has spread across the entire affected area, whereas the distribution of genotype A appears to be restricted to the northeastern part of the affected region. The diversity indexes (D) of the individual markers for the Dutch population, calculated according to Simpson (7), were 0.48, 0.12, 0.06, 0.00, 0.06, and 0.31 for Ms23, Ms24, Ms27, Ms28, Ms33, and Ms34, respectively,

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Address correspondence to C. H. W. Klaassen, c.klaassen@cwz.nl.

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TABLE 1 Clinical samples, geographical location, year of collection, and obtained MLVA genotypes from the Dutch Q fever outbreak episodes of 2007 to 2010[a]

Sample no., strain, or source	Clinical specimen	Geographical location	Yr	C_T value	No. of repeats						MLVA type
					Ms23	Ms24	Ms27	Ms28	Ms33	Ms34	
Q012	Urine	Balgoij	2008	31.7	6	11	3	3	2	7	A
Q013	Sputum	Beneden-Leeuwen	2008	31.9	6	11	3	3	2	7	A
Q014	Throat swab	Balgoij	2008	31.9	6	11	3	3	2	7	A
Q015	Plasma	Grave	2008	34.7	6	11	3	3	2	7	A
Q032	Serum	Wijchen	2008	31.7	6	11	3	3	2	7	A
Q033	Serum	Nijmegen	2007	30.9	6	11	3	3	2	7	A
Q034	BAL fluid	Wijk bij Duurstede	2008	26.6	6	11	3	3	2	7	A
Q007	Throat swab	Alverna	2008	31.9	6	11	3	3	2	8	B
Q018	Sputum	Balgoij	2008	34.2	6	11	4	3	2	7	C
Q008	Plasma	Nijmegen	2008	34.4	6	13	3	3	2	8	D
Q042	BAL fluid	Veldhoven	2009	29.9	3	11	3	3	2	8	E
Q084	Aorta valve	Zeeland	2008	17.0	3	11	3	3	2	8	E
Q102	Plasma	Nuenen	2010	29.1	3	11	3	3	2	8	E
Q072	BAL fluid	Prinsenbeek	2009	32.8	3	10	3	3	2	7	F
Q050	BAL fluid	Tilburg	2009	22.4	3	11	3	3	2	7	G
Q052	Sputum	Houten	2009	20.7	3	11	3	3	2	7	G
Q053	Sputum	Nieuwegein	2009	23.3	3	11	3	3	2	7	G
Q054	Sputum	Houten	2009	19.4	3	11	3	3	2	7	G
Q055	Sputum	Utrecht	2009	26.0	3	11	3	3	2	7	G
Q057	Sputum	Houten	2009	20.6	3	11	3	3	2	7	G
Q063	Sputum	Ravenstein	2009	29.6	3	11	3	3	2	7	G
Q064	BAL fluid	Eindhoven	2009	31.1	3	11	3	3	2	7	G
Q066	Sputum	Wijchen	2009	27.7	3	11	3	3	2	7	G
Q074	Wound fluid	Groesbeek	2009	NA	3	11	3	3	2	7	G
Q076	Aorta valve	Druten	2009	NA	3	11	3	3	2	7	G
Q078	Aorta valve	Standaardbuiten	2009	26.4	3	11	3	3	2	7	G
Q083	Thrombus	's-Hertogenbosch	2010	18.2	3	11	3	3	2	7	G
Q099	Abscess fluid	Son	2010	24.6	3	11	3	3	2	7	G
Q101	Vascular prosthesis	Handel	2010	22.7	3	11	3	3	2	7	G
Q103	Aorta tissue	Venlo	2010	32.3	3	11	3	3	2	7	G
Q104	Serum	Wijchen	2010	27.2	3	11	3	3	2	7	G
Q107	Aorta valve	Wijchen	2010	9.0	3	11	3	3	2	7	G
Goats ($n = 20$)	Placenta	Balgoij	2008	NA	3	11	3	3	2	7	G
Goat	Placenta	Wouda	2009	NA	3	11	3	3	2	7	G
Goat	Placenta	Denekamp	2009	NA	3	11	3	3	2	7	G
Q056	BAL fluid	Amersfoort	2009	28.2	4	11	3	3	3	8	H
Q011	Throat swab	Balgoij	2008	31.7	0	11	3	3	0	7	P
Q019	Urine	Balgoij	2008	36.8	0	11	0	3	0	4	p
Q020	Throat swab	Herpen	2008	38.3	0	0	0	3	0	0	p
Q021	Throat swab	Nijmegen	2008	37.8	0	0	4	3	0	0	p
Q022	Throat swab	Nijmegen	2008	37.9	0	0	4	3	0	0	p
Q067	Serum	Tilburg	2009	34.5	0	11	0	0	0	0	p
Q070	Serum	Tilburg	2009	32.0	0	0	0	0	0	7	p
Q075	Serum	Druten	2009	NA	0	11	0	3	0	0	p
Q079	Serum	Standaardbuiten	2009	NA	0	11	3	3	0	0	p
Q080	Aorta tissue	Standaardbuiten	2009	NA	0	11	3	3	0	0	p
Q089	Serum	Overloon	2010	34.8	0	0	3	0	0	7	p
Q098	Abscess fluid	Son	2010	28.3	0	11	3	3	0	7	p
Q100	Wound fluid	Handel	2010	30.5	0	0	3	3	0	7	p
C. burnetii Dugway					ND	5	4	4	3	3	
C. burnetii RSA 331					4	7	3	3	−1[b]	3	
C. burnetii RSA 493	DNA				9	27	4	6	4	5	
C. burnetii CbuG Q212					ND	8	3	4	2	2	
C. burnetii CbuK Q154					ND	9	4	5	2	2	

[a] The number of repeats in each marker was determined by extrapolation using the sizes of the obtained fragments relative to those obtained using DNA from the Nine Mile strain (RSA 493). Furthermore, the genotypes of four additional *C. burnetii* strains, i.e., Dugway (GenBank accession number CP000733), RSA331 (CP000890), CbuG Q212 (CP001019), and CbuK Q154 (CP001020) were determined *in silico* using the published sequences. NA, results not available; 0, no results obtained; p, partial genotype; ND, number of repeats could not be determined due to apparent sequence assembly errors.

[b] *In silico* analysis showed 5 fewer repeats than the Nine Mile strain (RSA 493), which by convention was assigned 4 repeats (1).

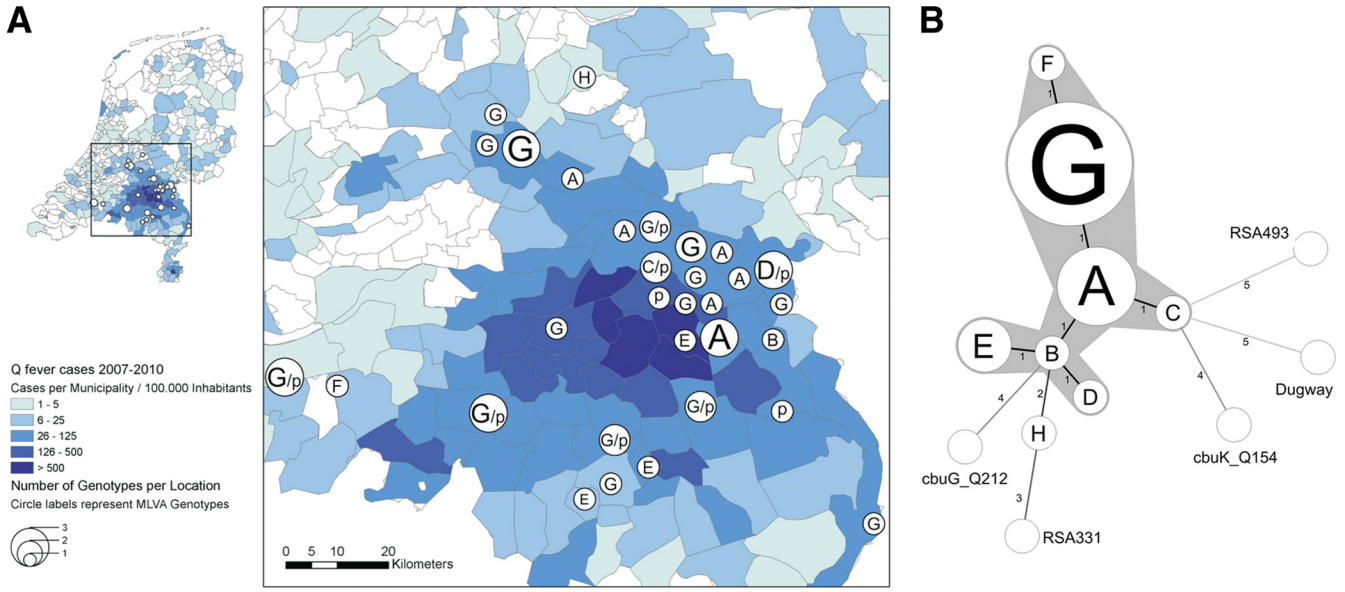

FIG 1 Geographical distribution and relationship between Dutch *C. burnetii* genotypes from humans. (A) Geographical locations of the MLVA genotypes obtained from the 2007-2010 outbreak. Syllables correspond to the genotypes identified in Table 1. (B) Minimum spanning tree showing the relationship between the obtained MLVA genotypes identified in this study. Only full MLVA genotypes obtained from clinical samples were included in this analysis. Additionally, the genotypes from five sequenced *C. burnetii* strains, i.e., Dugway (GenBank accession number CP000733), RSA331 (CP000890), Nine Mile RSA493 (AE016828), CbuG Q212 (CP001019), and CbuK Q154 (CP001020) were included. Each circle represents a unique genotype, and the size of the circle corresponds to the number of samples with that genotype. Branch labels and connecting lines correspond to the number of different markers between the genotypes. Genotypes connected by a gray background differ in only one marker from each other and may represent microvariants of one founder genotype.

versus 0.67, 0.79, 0.73, 0.74, 0.75, and 0.86, respectively, obtained from a reference collection of *C. burnetii* isolates from ticks, animal placenta, vaginal secretions, and milk and from human liver and blood (1). The D values observed in the Dutch population were much lower than in the reference population, indicating that the Dutch genotypes are much more closely related to each other and maybe arose from one founder genotype. The fact that multiple genotypes were obtained thus allows a certain degree of fine-structuring within the outbreak region.

Genotyping by using a 10-locus MLVA panel, including 4 out of the 6 markers used in our study, revealed one predominant MLVA genotype among goats and sheep throughout the affected Q fever area (5). In these samples, alleles were found in markers Ms24, Ms27, Ms28, and/or Ms34 that were identical to those in the human samples, implicating the goats and/or sheep as the most likely source of the outbreak. To substantiate this hypothesis, we included placenta samples from goats using all 6 markers from our MLVA panel. These goat samples were from 3 different locations in the outbreak area and contained the predominant ruminant *C. burnetti* genotype. MLVA genotype G was identified in all tested samples, illustrating the genotypic identity between *C. burnetii* from humans and goats. Compared to the 10-loci MLVA method, we chose the markers that seemed to be among the most discriminatory markers described (1), which enabled us to develop an easy-to-use and clearly distinguishable MLVA genotyping method.

Our results are consistent with a scenario where one MLVA genotype was introduced in the dairy animal population, where it is gradually diversifying over time while spreading over the country and being transmitted to humans. We believe that the sudden increase of Q fever infections in humans could have been facilitated by the expansion of intensive goat farming in the southeast of The Netherlands in the last 2 decades (4). Another possibility is that the Dutch *C. burnetii* isolates are from a hypervirulent lineage that may be disseminating more rapidly than other genotypes. Both explanations, however, require further investigation.

In conclusion, this study shows that the unprecedented and ongoing Q fever outbreak in The Netherlands involved not only multiple different but closely related MLVA genotypes found at several locations spread across the entire affected area during the Q fever outbreak years of 2007 to 2010, indicating a clonal spread of *C. burnetii* across The Netherlands.

REFERENCES

1. **Arricau-Bouvery N, et al.** 2006. Molecular characterization of *Coxiella burnetii* isolates by infrequent restriction site-PCR and MLVA typing. BMC Microbiol. **6**:38.
2. **Karagiannis I, et al.** 2009. Investigation of a Q fever outbreak in a rural area of The Netherlands. Epidemiol. Infect. **137**:1283–1294.
3. **Klaassen CHW, Nabuurs-Franssen MH, Tilburg JJHC, Hamans MAWM, Horrevorts AM.** 2009. Multigenotype Q fever outbreaks, the Netherlands. Emerg. Infect. Dis. **15**:613–614.
4. **Roest HIJ, et al.** 2010. The Q fever epidemic in the Netherlands: history, onset, response and reflection. Epidemiol. Infect. **5**:1–12.
5. **Roest HIJ, et al.** 2011. Molecular epidemiology of *Coxiella burnetii* from ruminants in Q fever outbreak, the Netherlands. Emerg. Infect. Dis. **17**: 668–675.
6. **Schimmer B, et al.** 2009. Sustained intensive transmission of Q fever in the south of the Netherlands, 2009. Euro Surveill. **14**:19210.
7. **Simpson EH.** 1949. Measurement of diversity. Nature **163**:688.
8. **Tilburg JJHC, et al.** 2010. Interlaboratory evaluation of different extraction and real-time PCR methods for detection of *Coxiella burnetii* DNA in serum. J. Clin. Microbiol. **48**:3923–3927.