Establishment of a genotyping scheme for *Coxiella burnetii*

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Abstract

Coxiella burnetii is the causative agent of Q fever. The bacterium is highly infectious and is classified as a category B biological weapon. The tools of molecular biology are of utmost importance in a rapid and unambiguous identification of *C. burnetii* in naturally occurring Q fever outbreaks, or in cases of a deliberate release of the infectious agent. In this work, development of a multiple locus variable number tandem repeats (VNTR) analysis (MLVA) for the characterization of *C. burnetii* is described. Sixteen *C. burnetii* isolates and five passage history/laboratory variants were characterized. The VNTR markers revealed many polymorphisms resulting in nine unique MLVA types that cluster into five different clusters. This proves that the MLVA system is highly discriminatory. The selected VNTR markers were stable. The MLVA method developed in this report is a promising tool for the characterization of *C. burnetii* isolates and their epidemiological study.

Introduction

Coxiella burnetii is an obligate intracellular, highly pleomorphic bacterium (Baca & Paretsky, 1983). It is highly infectious and causes Q fever, a zoonotic disease that is capable of transmission from animals to humans. Q fever infections occur worldwide with the exception of New Zealand (Hilbink *et al.*, 1993). Acute Q fever is a self-limiting febrile flu-like illness that can be resolved in a few weeks with antibiotics (Sawyer *et al.*, 1987). Chronic Q fever is usually presented as endocarditis or hepatitis (Stein & Raoult, 1995). *Coxiella burnetii* is extremely resistant to heat, desiccation, disinfectants and UV radiation (Madariaga *et al.*, 2003). Thus, it can persist in the environment under harsh conditions for long periods and can produce infection for weeks or months after exposure. The extremely low-infective dose of *C. burnetii* and its relative stability attribute to the listing of this organism as a category B biological weapon by the Center for Disease Control and Prevention, Atlanta, GA, USA (http://www.bt.cdc.gov) (Lederberg, 2000).

Identification of *C. burnetii* isolates by genotyping is a prerequisite for surveillance purposes and for epidemiological investigation in cases of natural outbreak or in deliberate release events. However, knowledge of the genetic heterogeneity in *C. burnetii* isolates is limited. The ribosomal RNA genes are conserved among isolates (Stein & Raoult, 1993). Pulsed field gel electrophoresis and a whole genome genotyping approach have classified the *C. burnetii* isolates into several groups (Heinzen *et al.*, 1990; Jager *et al.*, 1998). Moreover, the isolates have been differentiated into six genomic groups on the basis of DNA restriction fingerprints (Hendrix *et al.*, 1991). A limited heterogeneity was also detected in a few genes that were investigated (Zhang *et al.*, 1997; Nguyen & Hirai, 1999; Sekeyova *et al.*, 1999). However, it is difficult to compare information obtained by these various methods. Based on the published data and the fact that the *C. burnetii* genome has recently been sequenced (Seshadri *et al.*, 2003), a multiple locus variable number tandem repeats (VNTR) analysis (MLVA) genotyping approach seems to be feasible for this bacterium.

In this report, we describe an MLVA system that uses seven marker loci to discriminate *C. burnetii* isolates.

Materials and methods

Samples

In this study, the MLVA genotyping scheme has been developed using 16 isolates and five passage history/laboratory variants of the *Coxiella burnetii* isolates Nine Mile

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Table 1. Isolates/variants of *Coxiella burnetii*

	Isolation			
Isolate/variant	Country	Year	Source of isolation	Obtained from
NM-I RSA 493, EP_3	Montana, USA	1937	*Dermacentor andersoni* (tick)	Bratislava, Slovakia*
NM-I RSA 493	Montana, USA	1937	*Dermacentor andersoni* (tick)	Rijswijk, the Netherlands†
Unknown origin				Rijswijk, the Netherlands‡
NM-II RSA 439, EP_{165}	Montana, USA	1937	*Dermacentor andersoni* (tick)	Bratislava, Slovakia*
NM-II RSA 111			*Dermacentor andersoni* (tick)	Marseille, France§
1/IIA, EP_3	Slovakia	1968	*Dermacentor marginatus* (tick)	Bratislava, Slovakia*
DER, EP_3	Slovakia	1967	*Dermacentor marginatus* (tick)	Bratislava, Slovakia*
L, EP_3	Slovakia	1968	*Dermacentor marginatus* (tick)	Bratislava, Slovakia*
27, EP_5	Slovakia	1968	*Dermacentor marginatus* (tick)	Bratislava, Slovakia*
RAK8, EP_5	Tirol, Austria	1990	*Ixodes ricinus* (tick)	Bratislava, Slovakia*
IXO, EP_3	Slovakia	1957	*Ixodes ricinus* (tick)	Bratislava, Slovakia*
48, EP_3	Slovakia	1967	*Haemaphysalis punctata* (tick)	Bratislava, Slovakia*
Henzerling, EP_3	Italy	1945	Human blood, acute Q fever	Bratislava, Slovakia*
L35, EP_3	Slovakia	1954	Human blood, acute Q fever	Bratislava, Slovakia*
Florian, EP_5	Slovakia	1956	Human blood, acute Q fever	Bratislava, Slovakia*
S Q217	Montana, USA	1981	Human heart valve, endocarditis, chronic Q fever	Rijswijk, the Netherlands†
S, EP_3	Montana, USA	1981	Human heart valve, endocarditis, chronic Q fever	Bratislava, Slovakia*
Priscilla Q117	Montana, USA	1980	Goat placenta, abortion	Rijswijk, the Netherlands†
Priscilla, EP_3	Montana, USA	1980	Goat placenta, abortion	Bratislava, Slovakia*
LUGA, EP_3	Russia	1958	*Apodemus flavicollis* (mouse, spleen)	Bratislava, Slovakia*
Dugway 5J108-111	Utah, USA	1958	Rodent	Rijswijk, the Netherlands†

All isolates are in phase I except the NM-II isolates that are in phase II.

**Coxiella burnetii* lysates have been provided by Prof. Rudolf Toman (Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia).

†*Coxiella burnetii* DNA was kindly provided by Dr Martien Broekhuijsen (TNO Prins Maurits Laboratory, Rijswijk, the Netherlands) with permission of Dr Judith Tyczka (Institute for Hygiene and Infectious Diseases of Animals, Giessen, Germany).

‡The origin of this *C. burnetii* isolate kindly provided by Dr Martien Broekhuijsen and labeled as NM-I RSA is unknown.

§*Coxiella burnetii* DNA was kindly provided by Prof. Didier Raoult (Unité des Rickettsies, Marseille, France).

NM-I, Nine Mile phase I; NM-II, Nine Mile phase II; EP, egg passage.

(NM), Priscilla and S (Table 1). Materials were obtained as killed bacterial lysates (isolates from Bratislava) or DNA (isolates from Marseille and Rijswijk).

Tandem repeat search and primer design

The complete genome sequence of *C. burnetii* RSA 493 is known (Seshadri *et al.*, 2003) and available from Blast (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). This sequence was used for a search of tandem repeats and development of the primer sets for MLVA of *C. burnetii*. The whole genome sequence of the bacterium was screened for the presence of tandem repeats using the Tandem Repeats Finder software (Benson, 1999). From the list of results obtained, a selection of eight different loci was made. The selection was based on the following criteria: (1) the number of the repeats should be greater than 4; (2) the repeat size should not exceed 30 base pair (bp) (this criterion was included so as to be able to analyze the sizes of the tandem repeats on agarose gels); (3) the conservation among the repeats should be more than 90%. The most suitable repeats were selected and the primers were developed flanking these repeats using the primer developing program Kodon (Kodon 2.0 software, Total Genome and sequence analysis, Applied Maths, Sint-Martens-Latem, Belgium).

Multiple locus variable number tandem repeats analysis

Each VNTR locus was amplified using a forward primer labeled at the 5′ site with 6-carboxyfluorescein and an unlabeled reverse primer (Table 2). The PCR reactions were optimized for annealing temperature using a gradient DNA Engine™ Gradient Cycler apparatus [MJ Research® (Waltham, MA), PTC-200, Peltier Thermal Cycler] and the conditions were selected on the basis of highest product yield on agarose gels. The optimized PCR reactions were performed with an Applied Biosystems 9700 PCR apparatus (Foster City, CA). The PCR reaction (final volume 20 μL) included 10 μL of HotStar *Taq* master mix (QIAGEN, Hilden, Germany), 1 μL of each primer (10 pmol μL^{-1}), 6 μL of sterile water and 2 μL of DNA or lysate. The PCR program included 15 min of denaturation at 95 °C, followed by 25 cycles of amplification consisting of denaturation at 95 °C for 20 s, annealing for 30 s at a selected temperature (T_{ann}, Table 2) and elongation at 72 °C for 1 min.

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Table 2. Primer sequences, coordinates, annealing temperature, repeat size in base pairs and the nucleotide sequence of the repeat

Primers	Nucleotide sequence (5′–3′)	Genome coordinate	T_{ann} (°C)	Repeat length (bp)	Nucleotide sequence of repeat (5′–3′)	No. of repeats	No. of variants
#1 Cox 1F	FAM-AGAAAAAAGCACAGACCTTGA	1471821	53	6	GAAAAG	2–10	4
#1 Cox 1R	TTCCTGATTTAAAAGGGTGACT	1471952					
#2 Cox 2F	FAM-TTCTTTATTTCAGGCCGGAGT	838419	55	6	TGAAGA	2–4	3
#2 Cox 2R	CCGGTAACGCCGATTAGTAA	838581					
#3 Cox 3F	FAM-GCAATCCAGTTGGAAAGAA	831215	52	9	AGAAAATAA	2–18	6
#3 Cox 3R	ATTGAAGTAATCCATCGATGATT	831367					
#4 Cox 4F	FAM-ATGAAGAAAGGATGGAGGG	259502	53	21	GACAGAAGACGGAAG ACGGAA	2–9	4
#4 Cox 4R	TGCAAGGATAGCCTGGA	259854					
#5 Cox 5F	FAM-AATGGAGTTTGTTAGC AAAGAAA	839689	58	6	TAAGAA	3–7	5
#5 Cox 5R	AAAGACAAGCAAAACGATAAAAA	839841					
#6 Cox 6F	FAM-GACAAAAATCAATAGCCCGT	197645	53	7	GAGGACA	3–8	4
#6 Cox 6R	GAGTTGTGTGGCTTCGC	197796					
#7 Cox 7F	FAM-ACAGGCCGGTATTCTAACC	1418045	56	7	CAGAGGA	2–5	4
#7 Cox 7R	CCTCAGCACCCATTCAG	1418197					

T_{ann}, annealing temperature; bp, base pair.

Amplification was completed by incubation for 30 min at 68 °C to ensure a complete terminal transferase activity of the *Taq* DNA polymerase.

The PCR products obtained were diluted 100 times, and 2 μL of this dilution were mixed with 10 μL of 200 times diluted MapMarker Rox 400 Low (Eurogentec, Sering, Belgium). The samples were denaturated for 5 min at 95 °C and cooled on ice. 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 inputted into the Bionumerics 4.0 software package (Applied Maths). Each isolate was assigned by an MLVA profile, defined by the number of repeats found at the different VNTR loci. Each unique MLVA profile was assigned an MLVA type. To confirm both the accuracy of sizing determined by capillary electrophoresis and the translation of the fragment sizes into repeat numbers, the VNTR PCR fragments of all isolates and their variants were sequenced.

Data analysis

Clustering of the MLVA profiles was performed with BioNummerics 4.0 software using the unweighted pair-group method with arithmetic mean (UPGMA) and the categorical coefficient of similarity or using a graphical method called the minimum spanning tree; the categorical coefficient was used in the latter also (Pourcel *et al.*, 2004).

DNA sequencing

For DNA sequencing reactions, a fluorescence-labeled dideoxynucleotide technology from Applied Biosystems was used. Sequence reactions were analyzed on an ABI 3700 automated DNA sequencer. The sequences obtained were assembled and edited using Kodon 2.0 software.

Results

Identification of VNTR loci

Using the Tandem Repeat Finder software, eight sequences were selected that contained tandem repeats in the *Coxiella burnetii* genome. The length of the repeat varied from 6 to 21 bp. The primer sequences, named Cox 1–Cox 8, were designed and eight VNTR loci were tested on 21 *C. burnetii* samples. One of the eight loci was unsuitable for typing as no product was detected in any of the isolates or variants. The remaining seven VNTR primer sets were suitable for the MLVA typing and their characteristics are listed in Table 2. The human DNA did not yield a product using the seven VNTR PCRs.

The MLVA typing of 16 *C. burnetii* isolates and five passage history/laboratory variants using the seven selected VNTR markers revealed that the number of repeats varied between two and 18 repeats per VNTR locus and that the number of variant alleles per locus varied between three and six (Table 2). The nine unique marker allele size combinations (MLVA types) that were observed among the 21 *C. burnetii* samples were designated as A–I (Fig. 1). Sequencing of the VNTR PCR fragments showed a consequent difference of a single repeat unit that was found in excess with respect to the data found with GeneScan. In our work, the repeat number found with GeneScan was used and an inaccurate sizing was probably the result of the secondary structure in the PCR product.

Stability of the MLVA profiles

The stability of the chosen genetic markers was determined by analyzing the samples of NM isolate with different histories. Four NM variants with different numbers of egg passages (EP)

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Isolate/variant	Geno cluster	MLVA type	Source	Egg passage	Origin	VNTR locus #1	#2	#3	#4	#5	#6	#7
NM-II RSA (Marseille)	I	E	Tick		USA	5	4	4	9	6	8	5
NM-II RSA	I	E	Tick	165	USA	5	4	4	9	6	8	5
RAK8	I	E	Tick	5	Austria	5	4	4	9	6	8	5
NM-I RSA (Giessen)	I	E	Tick		USA	5	4	4	9	6	8	5
NM-I RSA	I	E	Tick	3	USA	5	4	4	9	6	8	5
Dugway (Giessen)	II	I	Rodent		USA	10	2	4	4	7	5	3
S	III	G	Man	3	USA	2	3	2	3	4	6	4
S (Giessen)	III	G	Man		USA	2	3	2	3	4	6	4
Priscilla	IV	F	Goat	3	USA	2	4	2	2	5	3	3
Priscilla (Giessen)	IV	F	Goat		USA	2	4	2	2	5	3	3
DER	V	B	Tick	3	Slovakia	3	3	18	2	3	3	2
IXO	V	B	Tick	3	Slovakia	3	3	18	2	3	3	2
LUGA	V	B	Mouse	3	Russia	3	3	18	2	3	3	2
1/IIA	V	A	Tick	3	Slovakia	3	3	13	2	3	3	2
Florian	V	A	Man	5	Slovakia	3	3	13	2	3	3	2
Henzerling	V	A	Man	3	Italy	3	3	13	2	3	3	2
L	V	A	Tick	3	Slovakia	3	3	13	2	3	3	2
48	V	C	Tick	3	Slovakia	3	3	14	2	3	3	2
27	V	D	Tick	5	Slovakia	3	3	13	2	3	3	5
L35	V	D	Man	3	Slovakia	3	3	13	2	3	3	5
Unknown (Rijswijk)	V	H				3	3	16	2	3	6	2

Fig. 1. Multiple locus variable number tandem repeats analysis clustering of the *Coxiella burnetii* isolates and their variants by the unweighted pair-group method with arithmetic mean categorical coefficient. For abbreviations, see Tables 1 and 2 or text.

in virulent phase I (NM-I) and low-virulent phase II (NM-II), stored at different laboratories, were all identical (Fig. 1). Our finding that RAK8 isolated in a different continent (Table 1) has the identical MLVA type as the NM isolate indicates that the same genotype is found or spread over the continents. In the latter case, this process might last for many years and thus it can be presumed that the RAK8 genotype is stable. Moreover, both NM and RAK8 isolates were isolated from ticks, and the finding of the same MLVA type could indicate migrations of infected ticks. The stability of the markers was further confirmed by the identical MLVA types for two S (MLVA type G) and two Priscilla (MLVA type F) isolates/variants that were stored at different laboratories (Fig. 1). Thus, all the data indicate stability of the genetic markers.

Genotyping of MLVA profiles

The UPGMA cluster analysis of the MLVA data revealed the existence of five major clusters (clusters I–V, Fig. 1). Cluster I consists of four NM variants, irrespective of their phase state, together with the RAK8 isolate. These were labeled MLVA type E (Fig. 1). Cluster II with MLVA type I consists of the Dugway isolate only. This isolate has one similarity with cluster I on the Cox 3 locus and one with cluster IV on the Cox 7 locus. Clusters III an IV consist of two S, MLVA type G, and two Priscilla, MLVA type F, respectively. Cluster V is more complex and harbors five different MLVA types (A, B, C, D and H). It mainly consists of acute Q fever- and tick-derived isolates. Based on MLVA typing, these isolates are closely related and differ one from another at most in two loci only.

Results of the UPGMA clustering of the MLVA data showed the genetic relationships among the MLVA profiles and grouping of the *C. burnetii* isolates and their variants into the different clusters. Clustering of the MLVA data using the minimal spanning tree graphing method gave a simpler representation of the genetic relations of the *C. burnetii* isolates in cluster V (Fig. 2). The lines represent relations among the MLVA types in the minimal spanning tree. The short solid lines represent a relation of six identical loci of the seven and the longer solid line of five identical loci of the seven. The dotted lines represent a very loose relationship (two or one of seven loci are identical). MLVA type A consists of four isolates, 1/IIA, Florian, Henzerling and L, and is central in the gene cluster. It is most likely a candidate for the origin of other types surrounding it. MLVA type B consists of the isolates DER, IXO and LUGA and has only one difference when compared with MLVA type A. This difference is at the Cox 3 locus where MLVA type B has 18 instead of 13 repeats (Fig. 1). MLVA type C contains the

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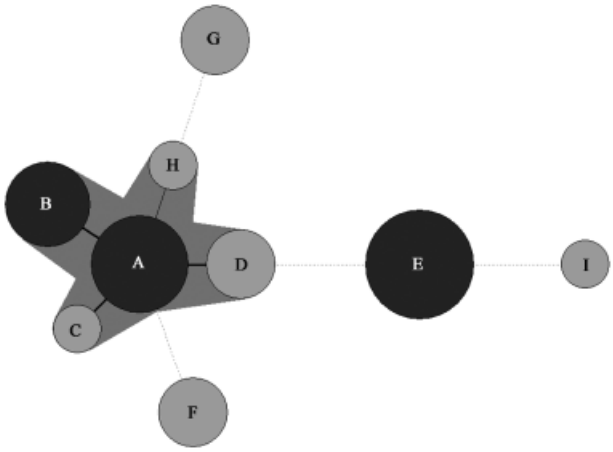

Fig. 2. Multiple locus variable number tandem repeats analysis (MLVA) genotypes of the *Coxiella burnetii* isolates and their variants using the minimal spanning tree graphing method. The gray circles represent one or two isolates/variants while the black ones represent three or more isolates/variants. The characters refer to the MLVA type. For abbreviations, see text.

isolate 48 and it differs only at the Cox 3 locus from MLVA type A. MLVA type D consists of the isolates 27 and L35 and these isolates differ in a single locus (Cox 7) from MLVA type A. MLVA type H contains an unknown isolate from Rijswijk with two differences at the Cox 3 and Cox 6 loci.

Discussion

This study shows that MLVA typing can be a reliable method for the characterization of *Coxiella burnetii* isolates and their passage history/laboratory variants. The VNTR markers used revealed many polymorphisms resulting in nine MLVA types in 21 *C. burnetii* samples. The markers are stable with time and independent of the phase state of the bacterium. It is assumed that this simple molecular tool will help unravel several interesting aspects of *C. burnetii* as for its molecular phylogeny and epidemiology when being applied to a larger number of isolates. The method is robust, simple, cheap, highly discriminatory, reproducible and portable. It can be used to create the isolate profiles that are easily electronically exchangeable. MLVA has been successfully used to type several different bacterial species and proven to be a good method with a high resolution (van Belkum *et al.*, 1997; Keim *et al.*, 1999; Coletta-Filho *et al.*, 2001; Farlow *et al.*, 2001, 2002; Klevytska *et al.*, 2001; Liu *et al.*, 2003; Pourcel *et al.*, 2003).

Using seven VNTR loci, 21 *C. burnetii* isolates and variants were investigated for length polymorphisms. The variations in a number of repeats, a number of variants of the repeats and in a number of the different MLVA types per number of isolates found in this work were similar to other studies (Schouls *et al.*, 2004; Top *et al.*, 2004). This implies that the chosen MLVA system has a high discriminatory capacity and is suitable for the molecular genotyping of *C. burnetii*.

Stability of the MLVA profiles is a prerequisite for a reliable molecular typing system. There are several indications of the stability of markers chosen in this study. Thus, four NM variants being in the different phase state (NM-I and NM-II) and maintained at the different laboratories (Bratislava, Giessen and Marseille) had the identical MLVA profile (type E) indicating stability of the chosen genetic markers. Similarly, the isolates S and Priscilla stored at the different laboratories were identical. In addition, the fact that the isolate RAK8 had an MLVA type similar to that of the NM variants is indicative of the stability of genetic markers. Finally, MLVA type D contained two isolates, 27 and L35, which were not related by the host or the date of isolation. Our finding that the isolate originally obtained as NM-I RSA from Rijswijk clustered into a different cluster than the NM isolate and its variants was surprising. After questioning the provider, it became evident that the isolate was of unknown origin. Thus, this finding has also proved the potential of the molecular typing method presented here in terms of its stability and usefulness.

The UPGMA clustering and the minimal spanning tree graphing method revealed the genetic relationships among the tested isolates and their variants. Both methods gave identical results. Roughly, five major clusters were apparent. The separation among the clusters was arbitrarily set at four identical loci per MLVA type. Cluster I, where the NM variants and the RAK8 isolate are present, differs clearly from other clusters. This might indicate their genetic isolation from other isolates/variants of the MLVA types G and F, and the tick-derived isolates. The large difference between the genocluster I tick group and other isolates that are derived from ticks and acute Q fever cases is worthy of further study. Nevertheless, it should be kept in mind that the preponderance of the genocluster I isolates is due to four of five isolates in this group being NM-I or passage history variants.

Our study also shows that there is a difference between the isolates Priscilla and S and those of acute disease and tick-derived isolates. This finding correlates with other data (Hendrix *et al.*, 1991; Nguyen & Hirai, 1999), where the difference among isolates from acute and chronic Q fever cases and tick-derived cases has been reported. Unfortunately, only two chronic Q fever-derived variants were available to our study, so we were unable to follow this topic in more detail. When the minimal spanning tree method was used, Priscilla and S were presented far from each other. However, both isolates were identical at two loci (Cox 1 and Cox 3) that are similar to their match with the MLVA types A and H, indicating that their location in the minimal

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spanning tree graph as for the related MLVA types is not ambiguous.

Cluster V consists of a genetically related group with the isolates from acute Q fever cases and ticks. This clustering has also been found by others (Hendrix *et al.*, 1991; Nguyen & Hirai, 1999) where the isolates from acute Q fever cases, cattle and arthropods clustered together. The minimal spanning tree graph gives a suggestion for the mutual relationships among the isolates. It shows that MLVA type A is central to the MLVA group. This implies that MLVA type A could be an evolutionary origin for other types surrounding it. This is, however, only a suggestion as only 16 isolates and five passage history/laboratory variants were available for this study. In future, the noncultured isolates from the field should be investigated in order to avoid a possible selection during the cultivation process, an observation that has been published recently for *C. burnetii* (Andoh *et al.*, 2004).

Most recently, Glazunova *et al.* (2005) have published a genotyping method for *C. burnetti* where multispacer sequence typing (MST) was used. A comparison of the MLVA UPGMA cluster analysis with MST shows that the results obtained by both methods are similar. However, when the MLVA method was applied, the NM isolate together with the Dugway and RAK8 isolates was clearly distant from other isolates. This suggests that the MLVA typing method might have a better discriminating capacity than the MST method. Further, it appears that the MLVA method has additional advantages over MST, although both methods gave similar results. The MLVA typing is less laborious and sequencing is not necessary, making the MLVA typing method robust, simple and even more portable than the MST method. This allows an easy and rapid exchange of data without errors that might occur when strains or isolates are sequenced. Moreover, the method is intended for direct use with the field material without the necessity of prior cultivation as it includes the amplification step and sensitive detection on GeneScan.

A limited number of *C. burnetii* isolates and their variants were available to this study, and more isolates will have to be tested in future to prove the potential of the method presented. Likewise, more isolates from acute and chronic Q fever cases should be examined in order to evaluate the method as a reliable tool for differential diagnosis of *C. burnetii* infections in humans.

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