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Molecular Epidemiology of Q Fever in Poland

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Abstract

Coxiella burnetii is the etiologic agent of Q fever, a worldwide distributed zoonosis, accountable for serious health problem both for humans and animals. The exposure to *C. burnetii* infected animals and their products is the main risk factor for Q fever in humans. Several outbreaks of Q fever have been described in Poland which sources were recognized to be related to imported animals and their products or to wildlife using serological methods. Moreover, some of them have been confirmed by isolation of *C. burnetii* strains. In this study, multispacer sequence typing (MST) and multiple loci variable number tandem repeats (VNTR) analysis (MLVA) have been used to characterize *C. burnetii* strains isolated in Poland. A total of two sequence types (MST) and four MLVA types were identified among 6 *C. burnetii* isolates examined. This study highlighted the usefulness of these methods in the improvement of epidemiological investigations of Q fever loci on the Polish territory.

Key words: *Coxiella burnetii*, MLVA, MST, molecular epidemiology

Introduction

Coxiella burnetii is the etiologic agent of Q fever. These bacteria are a strict obligate intracellular parasites bacterium recently included to the order *Legionellales*. This zoonotic disease is distributed in most vertebrates worldwide and it could pose a serious health threat for humans and animals. The exposure to *C. burnetii* infected animals and their products is regarded as the main risk factor for the occurrence of Q fever in humans. It can affect various occupational groups, professionally exposed to animals or their products, such as veterinarians, farmers and abattoir workers.

There were several outbreaks of Q fever reported in Poland from 1956 until 2005 (Gawron and Wagner, 1962; Lutynski, 1956; Tylewska-Wierzbanowska *et al.*, 1996; Tylewska-Wierzbanowska *et al.*, 1991). The sources of the outbreaks were recognized to be related to imported animals and their products. Infections were detected with serological methods, however some of them have been confirmed by isolation of *C. burnetii* strains.

Since the availability of whole genome sequences, bacteria were shown to possess a high percentage of repeated DNA motifs, structured in multiple copies evenly distributed throughout their genomes. Their study resulted in the establishment of variable number tandem repeats (VNTR) and its analysis (MLVA). MLVA is currently regarded as a valuable bacterial typing method, particularly suitable for molecular epidemiology. The MLVA is based on direct PCR amplification of the specific locus, which is well defined. The range and polymorphism index of each locus can be calculated (Arricau-Bouvery *et al.*, 2006; Monteil *et al.*, 2007; Pourcel *et al.*, 2007; Sue *et al.*, 2007; Svraka *et al.*, 2006; Tenover *et al.*, 2007; Torpdahl *et al.* 2007).

Additionally, multispacer sequence typing (MST), was proven to discriminate between *C. burnetii* strains, as it has been done for other species (Fournier *et al.*, 2004; Glazunova *et al.*, 2005; Paciorek *et al.*, 2006; Tylewska-Wierzbanowska *et al.*, 1991). Thirty four different sequence types of *C. burnetii* have been recognized by Glazunova *et al.* (2005). Some of them seems related to the acute form of the disease, while others to the chronic infection (Glazunova *et al.*, 2005).

In our study, MST and MLVA were used to characterize both human and animal Polish *C. burnetii* isolates.

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Experimental

Material and Methods

Bacterial strains. Six *C. burnetii* strains isolated in Poland, including 3 strains isolated from cattle placenta (Zam, 801, 507), one from bull semen (Dowg), 2 strains from human (755, Hum), Henzerling strain (collection of National Institute of Public Health – National Institute of Hygiene,Warsaw, Poland) and Nine Mile strain (collection of French Food Safety Agency, Sophia Antipolis, France) were analyzed (Table I).

Bacteria were cultured in HEL-299 (ATCC-CCL-137) human fibroblasts cells in a shell-vials with 5 ml of EMEM medium with Earle's BSS, 2 mM L-glutamine and supplemented with 10% fetal bovine serum. Infected cells were maintained in 5% CO_2 atmosphere at 35°C for 14 days.

DNA extraction. For DNA extraction, 200 µl of supernatant from each culture has been taken. DNA of *C. burnetii* bacteria was extracted with the QIAamp Tissue kit (QIAGEN Gmbh, Hilden, Germany). Concentration of extracted DNA was measured as the optical density with BioPhotometer (Eppendorf, Hamburg, Germany) at 260 nm. DNA samples were stored at –20ºC.

MST. Extracted DNA was amplified with primer pairs Cox18, Cox51, Cox20, Cox37, Cox57, Cox61. All sequences of primers were described by Glazunova *et al*. (2005).

The reaction mixtures of 50 µl contained 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% gelatin, 200 µM dNTPs, 50 pmol of each primer and 1.5 U Taq DNA polymerase (Perkin-Elmer Cetus, USA). An aliquot of 5 µl of DNA template (approximately 100 ng/µl) was added to each reaction mixture. The cycling conditions were as follows: 3 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 57ºC, 1 min at 72°C and finally 7 min at 72°C. PCRs were performed in a Mastercycler gradient apparatus (Eppendorf AG, Germany). Each run of PCR test included positive (DNA from *C. burnetii* Nine Mile strain and Henzerling strain) and negative template (water). All amplicons were analysed by electrophoresis in 1.5% agarose gel stained with ethidium bromide.

Following amplification, PCR products were purified using the QIAquick PCR purification kit (QIAGEN Gmbh, Hilden, Germany) according to the manufacturer's protocol with modification. DNA was eluted with deionised water, instead of 10 mM Tris-Cl (pH 8.5) buffer.

All amplicons were sequenced with the ABI 377 DNA Analyzer (Applied Biosystem, USA) according to the manufacturer's recommendations. Sequences were edited using the Autoassembler software (Applied Biosystem, USA) and identified using the BLAST software by comparison with sequences available in GenBank.

After sequencing and assignment of allele numbers to all six loci (allele number and nucleotide sequence for all the identified alleles for each locus are made freely available online, by Glazunova *et al*., for comparisons to be made: http://ifr48.timone.univ-mrs.fr/MST_Coxiella/mst/group_detail), each strain was then designated by a combination of six numbers, called an allelic profile, which represented a sequence type (ST) for the particular strain. Subsequent isolates with an identical allelic profile were assigned to the same ST number and considered to be isogenic as they were indistinguishable at all six loci.

MLVA. Seventeen different VNTR loci (Table II) consisting of two panels were selected for DNA amplification as described by Arricau-Bouvery *et al.* (2006) and Svraka *et al*. (2006).

The reaction mixtures of 25 µl contained: 1 × PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µmol of each primer and 1.25 U of Platinum *Taq* DNA Polymerase (Invitrogen, USA). An aliquot of 2 µl (10 ng) of DNA template was added to each reaction mixture.

The cycling conditions were as follows: 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 58,5°C, 1 min at 72°C and finally 5 min at 72°C. The PCR products were run on 4% high resolution agarose gel or 1% agarose gel (for amplified products from

Table I
MST types of Polish *C. burnetii* strains

Strain	Origin (year of isolation)	Country (state/town)	ST type	Alleles of 6 spacers					
				Cox18	Cox51	Cox20	Cox37	Cox57	Cox61
Nine Mile (ref. strain)	Tick (1935)	USA (Montana)	ST16	5	6	3	1	6	5
755	Human blood (1956)	Poland (Gorlice)	ST16	5	6	3	1	6	5
Hum	Human urine (1991)	Poland (Warsaw)	ST18	1	7	6	4	6	3
Zam	Cattle placenta (1983)	Poland (Zamość)	ST18	1	7	6	4	6	3
801	Cattle placenta (1993)	Poland (Leszno)	ST18	1	7	6	4	6	3
507	Cattle placenta (1993)	Poland (Leszno)	ST18	1	7	6	4	6	3
Dowg	Bull semen (1989)	Poland (Koszalin)	ST18	1	7	6	4	6	3
Henzerling (ref. strain)	Human blood (1945)	Italy	ST18	1	7	6	4	6	3

loci ms07 and ms12 only) with 100 bp size marker (Invitrogen, USA).

Differences in PCR products were analysed with Quantity One – 1D Analysis Software (BIORAD Laboratories). For ease of interpretation and number of repeat units estimation two reference control strains (Nine Mile and Henzerling, for which the expected MLVA pattern is known) were included in all the experiments (Arricau-Bouvery *et al.* 2006; Svraka *et al*. 2006).

Results

MST. Amplification yielded products with all primer pairs (Cox18, Cox51, Cox20, Cox37, Cox57, Cox61). All PCR products were sequenced which allowed the identification of 2 sequence types (ST). Strain 755 and Nine Mile have been assigned to belong to ST16 while Henzerling, 801, 507, Zam, Dowg, Hum strains to ST18. Allele numbers of the tested spacers are shown in Table I.

MLVA. The MLVA typing of the tested *C. burnetii* strains revealed that the number of repeats assayed at 17 different loci (two panels) varied between 2 to 27. The observed number of variant alleles was 1 in 5 loci, 2 in 11 loci and 3 in one locus (Table II).

The analysis of the number of repeats obtained using panel I and II markers revealed identity of strain 755 (isolated from human blood) to *C. burnetii* Nine Mile at all loci, except for locus ms27. In the strain 755 locus ms27 represents 3 instead of 4 copies of the repeat unit.

The 5 remaining strains and Henzerling strain were identical to them at loci: ms01, ms03, ms07, ms21 and ms22 with the same number of repeats. However, they differed from them at loci ms20, ms26, ms30 and ms36 from panel I and at all 7 loci from panel II (ms23, ms24, ms27, ms28, ms31, ms33 and ms34) as shown in Table II.

Moreover, the patterns of strains 801, 507, Zam, Dowg, Hum and Henzerling were identical to each other at all loci. Those isolates could be further discriminated, as indicated by the number of repeats within locus 12. Isolates clustered as follows: the first cluster included two isolates from cattle placentas, the second one included one isolate from cattle placenta and one from bull semen and the third one contained a single strain isolated from human blood and the strain Henzerling.

Discussion

Two MST (sequence types) and four MLVA profiles (indicated as B, C, D and E) were identified among the 6 Polish *C. burnetii* isolates examined. MST and MLVA typing have shown only slight heterogeneity of Polish isolates originating from different sources and regions.

The first epidemic of Q fever in Poland was detected in Gorlice (south part of Poland) in year 1956 (Lutynski, 1956). The source of infection for farm workers were sheep imported from Romania. Few months later samples of wool collected from these sheep were the source of infection for laboratory employees at the Institute of Zootechnics in Kraków (Lutynski, 1956). The strain no 755 was isolated, in 1956, from blood of a human with acute Q fever (acute onset of illness headache, fever 40°C, myalgia and dry cough). VNTR patterns of Nine Mile and 755 were similar except ms27 allele (Table II). These strains both belonged to ST16. All *C. burnetii* strains isolated later were classified into ST18. Their MLVA typing also revealed some other differences.

Despite of their different geographic origin and time of isolation strains Zam and Dowg had the same MST and VNTR patterns. Strain Zam was cultured from cattle placenta in eastern Poland in 1983. In this region the Q fever was recognized in humans and animals. The source of infection was never established and epidemiological investigations failed to provide an evidence of imported animals or their products. Strain Dowg was isolated from bull semen on a cattle farm in northern Poland in 1992.

Isolates 507 and 801 were recovered from two cattle placentas near Leszno in 1993 (Tylewska-Wierzbanowska 1996). These two share exactly the same MST and VNTR patterns. First time aborting cows were found in this region in 1988. Spread of *C. burnetii* infection among cattle herds and among humans in this region and the fact that there was no imported animals, might suggest that semen used for artificial insemination was a potential source of infection.

Strain Hum, was isolated from a child (boy, ten years old) with co-infection: mycoplasmosis (confirmed by serology) and Q fever. He presented a mild pneumonia with cough and fever. His chest radiograph showed nonspecific changes. Source of infection was never detected in this case. It is known, however, that this child spent his vacation abroad. The isolated strain has shown similarity to *C. burnetii* Henzerling strain. Obtained results suggest that the origin of strain 755 and five other tested strains is different.

The analysis with MST and MLVA of isolates from different outbreaks of Q fever showed their genotypic heterogeneity. Strain 755 represent unique VNTR and MST patterns otherwise never recognized later among strains isolated in Poland. It may suggest that this isolate was imported and effectively eliminated due to sanitary intervention and elimination of infected animals. As the result of those actions, this particular strain has not spread later on Polish territory, even

Table II
MLVA results of *C. burnetii* strains isolated on Polish territory

Strain	Origin (Year)	Country (Town)	MLVA patterns																MLVA type
			Panel I										Panel II						
			ms01	ms03	ms07	ms12	ms20	ms21	ms22	ms26	ms30	ms36	ms23	ms24	ms27	ms28	ms31	ms33	ms34	
Nine Mile (ref. strain)	Tick (1935)	USA (Montana)	4.0	7.0	8.0	**8.0**	15.0	5.0	6.0	4.0	6.0	7.0	8.0	27.0	**4.0**	6.0	5.0	9.0	5.0	A
755	Human blood (1956)	Poland (Gorlice)	4.0	7.0	8.0	**8.0**	15.0	5.0	6.0	4.0	6.0	7.0	8.0	27.0	**3.0**	6.0	5.0	9.0	5.0	B
801	Cattle placenta (1993)	Poland (Leszno)	4.0	7.0	8.0	**8.0**	19.0	5.0	6.0	16.0	5.0	16.0	3.0	8.0	**3.0**	3.0	2.0	5.0	4.0	C
507	Cattle placenta (1993)	Poland (Leszno)	4.0	7.0	8.0	**8.0**	19.0	5.0	6.0	16.0	5.0	16.0	3.0	8.0	**3.0**	3.0	2.0	5.0	4.0	C
Zam	Cattle placenta (1983)	Poland (Zamość)	4.0	7.0	8.0	**7.0**	19.0	5.0	6.0	16.0	5.0	16.0	3.0	8.0	**3.0**	3.0	2.0	5.0	4.0	D
Dowg	Bull semen (1989)	Poland (Koszalin)	4.0	7.0	8.0	**7.0**	19.0	5.0	6.0	16.0	5.0	16.0	3.0	8.0	**3.0**	3.0	2.0	5.0	4.0	D
Hum	Human urine (1991)	Poland (Warsaw)	4.0	7.0	8.0	**6.0**	19.0	5.0	6.0	16.0	5.0	16.0	3.0	8.0	**3.0**	3.0	2.0	5.0	4.0	E
Henzerling (ref. strain)	Human blood (1945)	Italy	4.0	7.0	8.0	**6.0**	19.0	5.0	6.0	16.0	5.0	16.0	3.0	8.0	**3.0**	3.0	2.0	5.0	4.0	E

though it is known that *C. burnetii* strains with ST16 profile are encountered in Europe (Romania, Germany and France) as well as on other continents (Cracea, 1987; Glazunova, 2005). Results of the study show that all *C. burnetii* strains isolated in Poland after the 1956's, between the sixties and the eighties outbreak and representing ST18 have been imported with animals and their products. It is known that this sequence type as well as ST16 correlates with acute form of the disease. The occurrence of strains with such pathogenic phenotype may explain why chronic Q fever cases have not been recognized until now in Poland.

Polymorphism obtained by MLVA typing allowed to detect genetic heterogeneity of Polish isolates originating from different sources and regions with a good discriminatory power. This study confirmed that this technique may be used in the future for epidemiological investigations of Q fever foci on the Polish territory. The obtained MLVA types C, D and E are different from MLVA patterns of strains from France and it seems that they are closer related to strains from Austria and Germany (Arricau-Bouvery *et al.* 2006; Svraka *et al*. 2006). Information embedded in the allele frequencies might give clues to the origin and source of isolates.

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