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Genotyping of *Coxiella burnetii* detected in placental tissues from aborted dairy cattle in the north of Algeria

M. Rahal[a,b], D. Tahir[a], C. Eldin[a], I. Bitam[a,b], D. Raoult[c], P. Parola[a,*]

[a] Aix-Marseille Univ, IRD, SSA, AP-HM, VITROME, IHU Méditerranée Infection, Marseille, France
[b] School of Veterinary Sciences, El Harrach, Algeria
[c] Aix-Marseille Univ, IRD, AP-HM, MEPHI, IHU Méditerranée Infection, Marseille, France

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ABSTRACT

Coxiella burnetii, is an obligate intracellular bacterium which is present throughout the world. In humans, *C. burnetii* is the causative agent of Q fever. In cattle, the infection is suspected to cause stillbirths, retained fetal membranes, metritis and infertility. The birth products of ruminants shed huge amounts of bacteria, and are considered a major source for human infection. The present study was designed to search for the presence of *C. burnetii* in placental tissues collected from aborted and normal calving dairy cows in Algeria, using molecular tools.

A total of 77 placental tissue fragments were collected from dairy cows. 73 samples were collected from aborted cows and four samples were collected from natural calving cows over a period of two years from January 2013 to March 2015. The presence of *C. burnetii* in these samples was screened by quantitative real-time polymerase chain reaction (qPCR) targeting two different genes, IS1111 and IS30 A. The positive PCR amplicons were subsequently sequenced for Multispacer Sequence Typing determination (MST) using seven pairs of sequences (Cox2, Cox5, Cox18, Cox37, Cox56, Cox57, and Cox61).

Fourteen placental tissues (19.1%) were found to be positive for *C. burnetii* by qPCR; 9 (12.3%) from the city of Blida and 5 (6.84%) from the city of Medea. Genotyping of the corresponding amplicons displayed 100% identity with *C. burnetii* MST20 genotype, confirming the circulation of this clone in dairy farms from Algeria.

1. Introduction

Coxiella burnetii is a small bacterium, which is 0.2–0.4 μm wide and 0.4–1 μm long. It is an intracellular pathogen which replicates in eukaryotic cells [1]. Although *C. burnetii* has a cell wall similar to that of Gram-negative bacteria, it is not stainable using the Gram technique. The Gimenez method is, therefore, used to stain *C. burnetii* isolated in culture [1]. *C. burnetii* is the agent of Q fever, a zoonosis first described in Australia in 1937 [2]. This bacterium can survive for very long periods in dust and infects a wide range of animals, from arthropods to humans [2].

The main reservoirs of *C. burnetii* are domestic ruminants [1]. The inhalation of aerosols infected with *C. burnetii* is the most common route of human infection [1]. It can occur after direct exposure to infected animals and their products and infection may occur especially at times of parturition or slaughtering. In non-pregnant animals, *C. burnetii* infection is most often asymptomatic [3]. In goats and sheep, it is a well-known cause of abortion, stillbirths, premature delivery, and weak offspring [4]. In cattle, detection of *C. burnetii* in animals with reproductive disorders (*eg.* premature delivery, stillbirth, infertility, retained fetal membranes, metritis and mastitis) have been reported [3–5]. However, the causality link is controversial, because of the absence of description of tissular corresponding lesions in most of the studies [6,7]. Recently [8], have found mild to severe endometritis in 10 *C. burnetii* PCR positive cow uterine biopsies. In the same work, the authors reported for the first time the presence of intralesional and intracytoplasmic *C. burnetii* in macrophages of the endometrium, providing some evidence for the role of this pathogen in reproductive disorders in cattle.

C. burnetii multispacer sequence typing (MST) is a typing method based on the sequence of intergenic spacers located between ORFs [9,10] The first description of this method by Glazunova et al. allowed identifying 30 different genotypes and three monophyletic groups among 173 *C. burnetii* isolates. Since this first work, other studies have used this technique, implementing the lists of *C. burnetii* genotypes around the world [1]. Some MST genotypes are cosmopolitan and occur in the five continents, as is the case of MST20 and MST16. MST8 has been described in two continents, Europe and North America [10],

* Corresponding author at: VITROME, IHU Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France.
E-mail address: philippe.parola@univ-amu.fr (P. Parola).

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while others are very specific to a single country, such as MST41 and MST49 (only described in France) [11], and MST17, which is the only circulating genotype in French Guiana [12]. Concerning host specificity, some MST genotypes have only been identified in human samples, such as MST5, which has been exclusively identified from the human heart valve [10]. Other MSTs have been identified from different host species, such as MST16 which has been found in samples taken from humans, ruminants and even arthropods [10].

In Algeria, human Q fever infection is not a notifiable disease. Therefore, official data on the incidence of cases are not available from the Ministry of Health, Population and Hospital Reform (*Ministère de la Santé, de la Population et de la Réforme Hospitalière*). In addition, although abortion of infectious origin is considered a real problem in dairy cattle farming because of its economic loss, the declaration and investigation of cases are not mandatory in this country and no study has been conducted on the prevalence of *C. burnetii* around abortions in cattle. Simultaneously, the circulating MST genotypes of *C. burnetii* are still unknown in North Africa, including Algeria. Our aim was to investigate the presence of *C. burnetii* and the circulating genotypes in the placentas of aborted and naturally calving cattle in northern Algeria.

2. Materials and methods

2.1. Study site and sample collection

The study was conducted in four Wilaya (provinces) of the northern region of Algeria, namely Blida (36° 28′ 0.12″ N, 2° 49′ 0.01″ E), Medea (36° 16′ 0.12″ N, 2° 45′ 0″ E), Bouira (36° 21′ 59.98″ N, 3° 52′ 59.99″ E) and Bordj-Bou-Arreridj (36° 4′ 0″ N, 4° 46′ 0″ E). These areas (Fig. 1) are an important dairy basin with approximatively 80,822 dairy cattle (Annex 1).

Between 2013 and 2015, a total of 77 placenta fragments were sampled, of which four were from naturally calving cows and 73 from aborted cows within 12–24 hours after the abortion. Abortion was defined as a loss of the fetus between the age of 42 days and approximately 260 days. In order to minimize the risk of contamination during the collection process, the samples have been collected at the same time as manual extraction of placenta, before contact with soil or feces. When the calving took place before the arrival of the veterinarian, the sampled placental tissues were washed at least twice in distilled water to remove dirt. Each sample ($\approx 25\ cm^3$) was individually transferred to 10 mL vials containing 70% ethanol until DNA extraction.

2.2. Sample processing

All samples were transferred for analysis to IHU Mediterranée Infection, in Marseille, France. Prior to DNA extraction, each placental fragment was rinsed twice in sterile water and dissected into small pieces using scalpel blades and then crushed manually using pestle. About 40 mg of each homogenate was digested with 25 µL of proteinase K and 180 µL of buffer G2 at 56 °C for 16 h. Total DNA was extracted in a final volume of 100 µL from each sample using the commercial EZ1® DNA Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The DNA was stored at −20 °C under sterile conditions to preclude contamination until the sample was used for PCR and genotyping. All DNA samples were individually screened for the presence of *C. burnetii* by specific real-time PCR with primers and probes designed to amplify IS1111 [13]. Subsequently, positive results were confirmed by the highly *C. burnetii*-species specific IS30 A, as previously described [14] (Table 1). *C. burnetii* DNA extracted from bacterial culture of *C. burnetii* Nine Mile (Tick, USA) MST16 was used as positive control. DNA-free water was also included in each reaction to control for possible contamination during the preparation of the mix. The results were considered positive when the cycle threshold (Ct) was inferior to 35 for the two different *C. burnetii* specific genes. Estimates of the correspondence between Ct of IS1111 qPCR and number of copies of the *C. burnetii* genome was performed using a correlation curve previously described [15]. All placental tissues were screened for two other bacterial pathogens involved in abortions in cattle: *Chlamydia* spp. and *Leptospira* spp. targeting the 23S rRNA and rrs (16 S) genes respectively. [16,17].

2.3. Multispacer sequence typing and sequence analysis

All qPCR positive samples for *C. burnetii* (14 cases) were used to determine the genotypes by MST. The spacer regions in the *C. burnetii* genome that exhibit higher variation for differentiating the genotypes (Cox2, Cox5, Cox18, Cox37, Cox56, Cox57, and Cox61) were selected for standard PCR using similar PCR conditions as previously described [10] (Table 2). All amplicons were purified using the PCR filter plate Millipore Nucleo Fast 96 PCR kit (Macherey Nagel, Düren, Germany) in line with the manufacturer's recommendations. The sequence and program reaction were carried out according to the instructions previously described [17]. Finally, all sequences generated were assembled and corrected on ChromasPro 1.7 software (Technelysium Pty Ltd., Tewantin, Australia). Sequence types were determined using the MST database and previous publications [18,15].

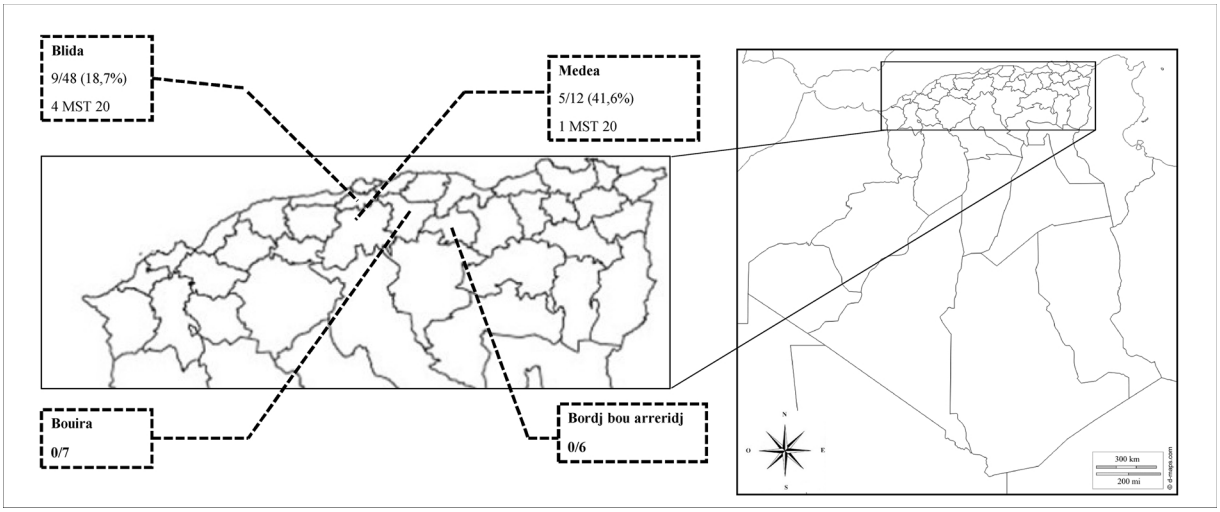

Fig. 1. Map of Algeria showing the geographical distribution of positive samples and according found MST genotypes of *C. burnetii*.

Table 1
Sequence of primers and probes used in the study for real-time PCR screening for the presence of *Coxiella burnetii*, *Chlamydia* spp. and *Leptospira* spp. DNA.

Target bacteria	Target sequence	Orientation	Nucleotide sequence (5′–3′)
Coxiella burnetii	Spacers	Forward	CAAGAAACGTATCGCTGTGGC
	IS1111	Reverse	CACAGAGCCACCGTATGAATC
		/	6FAM-CCGAGTTCGAAACAATGAGGGCTG-TAMRA
Coxiella burnetii	IS30A	Forward	CGCTGACCTACAGAAATATGTCC
		Reverse	GGGGTAAGTAAATAATACCTTCTGG
		/	6FAMCATGAAGCGATTTATCAATACGTGTATGC-TAMRA
Chlamydia spp.	Ch23S-F	Forward	CTGAAACCAGTAGCTTATAAGCGGT
		Reverse	ACCTCGCCGTTTAACTTAACTCC
		/	6FAM-CTCATCATGCAAAAGGCACGCCG-TAMRA
Leptospira spp.	rrs (16S)	Forward	CCCGCGTCCGATTAG
		Reverse	TCCATTGTGGCCGRA/GACAC
		/	FAM-CTCACCAAGGCGACGATCGGTAGC- TAMRA

2.4. Ethical considerations

The collection of samples from cows was carried out in accordance with Algerian legislation. Permission was obtained from the *Wilaya Veterinary Inspectorate* of each department.

3. Results

3.1. Detection of C. burnetii by quantitative real time-PCR (qPCR)

Of the 73 samples collected in four departments, Blida (N = 48), Medea (N = 12), Bouira (N = 7) and Bordj-Bou-Arreridj (N = 6), 14 samples (19.1%) tested positive by IS1111 qPCR including nine in Blida and five in Medea. Of these positive samples, two were highly loaded with *C. Burnetii* with a Ct value lower than 22 (16.2 and 21.2 corresponding to 9.0 and 7.5 log10 number of DNA copies/mL per sample, respectively). While all the others were moderately loaded with Ct values ranging between 28 and 33 (corresponding to 5.3 and 4.1 log10 of DNA copies/mL per the sample, respectively). All samples collected in Bouira and Bordj-Bou-Arreridj (N = 13) were negative (Fig. 1). Samples collected after natural calving displayed negative results. qPCR testings for the presence of *Chlamydia* spp. and *Leptospira* spp. were negative for all samples.

3.2. Multispacer sequence typing

The fourteen IS1111 qPCR-positive samples were sequenced. All these samples showed qPCR IS1111 Ct values lower than 29, corresponding to 5.1 log10 of DNA copies/mL. Of the seven spacers (Cox2, Cox5, Cox18, Cox37, Cox56, Cox57, and Cox61) targeted for sequencing, at least one exploitable sequence was obtained for each sample (Tables 3). The ST was successfully determined for only five samples (Table 3) because we successfully obtained complete sequences for the indispensable spacers (Cox2, Cox5 and Cox18) only for these five samples. Each one of these five sequenced samples showed the MST 20 genotype from placental tissues collected in Blida (N = 4) and Medea (N = 1). Concerning the nine failed attempts to ST determination (Table 3) because of uncomplete sequences, the individual comparative analysis of the obtained sequences from the Cox37 showed 100% sequence similarity within them and were 100% (417/417 bp) identical with the *C. burnetii* strain RSA331 (GenBank accession CP000890). The Cox56 sequences (samples nos. 39 and 73) showed 100% (384/384 bp) identity to the *C. burnetii* strain CB88 (GenBank accession AY502783). The obtained sequences for Cox61 (samples nos. 11 and 39) showed 100% (608/608 bp) sequence similarity with the *C. burnetii* strain RSA439 (GenBank accession CP018005). Finally, the sequence of the Cox57 spacer (sample 73) was 100% (598/598 bp) identical with the *C. burnetii* strain RSA439 (GenBank accession CP018005).

4. Discussion

In this study, we found that qPCR targeting the *C. burnetii* IS1111 sequence from placental tissues was positive in 19.1% of aborted cows. The positive cases were detected in two areas; Blida and Medea. All samples from Bouira and Bordj-Bou-Arreridj had negative results. However, the low number (N = 15) of collected samples may represent a bias and explain this negative result. Moreover, the sampling was based only on incidental cases of abortion in the study area, so that the sample size depended closely to new cases of abortions during the study period. Also, farmers were less likely to warn the veterinarians when their cows were going to drop a calf without complications. This can explain the limited number of samples from normal calving cows. In Algeria, veterinary services focus on brucellosis as a cause of abortion, thanks to a multiannual national screening program, which is based on the Rose-Bengal and the complement fixation test as a confirmatory testing [19]. As a consequence, we hypothesized that our sampled cows

Table 2
Primer sequences used for *Coxiella burnetii* genotyping.

Spacer name	Open Reading Frame (ORF)	Nucleotide sequence (5′–3′)	Amplified fragment length (bp)
Cox2	Hypothetical protein		397
	Hypothetical protein	GAAGCTTCTGATAGGCGGGA	
		CAGGAGCAAGCTTGAATGCG	395
		TGGTATGACAACCCGTCATG	
Cox18	Ribonuclease H	CGCAGACGAATTAGCCAATC	557
	DNA polymerase III, epsilon subunit	TTCGATGATCCGATGGCCTT	
Cox37	Hypothetical protein		463
	Hypothetical protein	ATTCCGGGACCTTCGTTAAC	
Cox56	OmpA-like transmembrane domain protein		479
	Conserved hypothetical protein	ATGCGCCAGAAACGCATAGG	
Cox57	Rhodanese-like domain protein		617
	Hypothetical protein	GGTTGGAAGGCGTAAGCCTTT	
Cox 61	Dioxygenase, putative	GAAGATAGAGCGGCAAGGAT	611
	Hypothetical protein	GGGATTTCAACTTCCGATAGA	

Table 3
Multispacer Sequence Typing (MST) found in positive samples.

Sample ID (N = 14)	Selected spacers							MST groups
	COX2	COX5	COX18	COX37	COX56	COX57	COX61	
10	–	–	–	+	–	–	–	–
11	–	–	–	+	–	–	+	–
12	–	–	–	+	–	–	–	–
17	–	–	–	+	–	–	–	–
31	–	–	–	+	–	–	–	–
40	–	–	–	+	+	–	+	–
72	–	–	–	–	+	+		–
74	–	–	–	+	–	–	–	–
75	–	–	–	+	–	–	–	–
20, 25, 30, 39 and 73	COX2.**3**	COX5.**2**	COX18.**6**	COX37.**4**	COX56.**10**	COX57.**6**	COX61.**5**	**20**

+ : sequenced; - : failed; 20, 25, 30, 39 and 73: samples in which MST genotyping was successfully determined; 3, 2, 6, 4, 10, 6, and 5: numbers corresponding to each amplified spacers.

were brucellosis free. However, other infectious agents may have been neglected.

In this study, we detected the presence of *C. burnetii* MST20 in placentas from aborted dairy cows, which has never been reported in Algeria to date. We used MST genotyping to determine *C. burnetii* genotypes [10]. This method is very discriminant and has been used in 19 different studies detecting *C. burnetii* in animals, humans or in the environment around the world [1]. This method is directly applicable to different sample types without the need for enrichment by a culture step comparing to the whole genome sequencing techniques. Also, a worldwide database of MST is available, allowing interlaboratory and geographical comparison of genotypes propagation. For this reason, it has been called a “geotyping method”. In comparison to other genotyping methods, it is less discriminant than Multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA), which is another method that has been predominantly used in Europe among ruminants [1]. However, we didn’t choose to use this method because it lacks interlaboratory reproducibility and analyze relatively instable repetitive elements than can produce too discriminatory results. The other existing methods has been used in too small or local settings, explaining why we didn’t use them (RADP, adaA, SNP) [1].

In Algeria *C. burnetii* has already been reported in small ruminants and *C. burnetii* DNA was detected in 57 (21.3%) vaginal swab samples from a total of 267 sheep and goats flocks [20]. This value is close to our estimated prevalence (19. 1%). Very often, farmers breed sheep, goats and cows in the same farm and they share the same pasture areas. This may be the main reason for contamination between these ruminants. Human cases of infective endocarditis caused by *C. burnetii* have already been detected in Algeria and Lacheheb and Raoult [21] found a seroprevalence rate of 15.5% in this country (113/ 729) in humans. Seroprevalence is estimated to be 18.5%, with a wide variation from 7.7% in urban areas to 35% in rural areas. The presence of *C. burnetii* in aborted cattle may be a source of human infection. In Europe, genotyping studies have shown that sheep and goats were the main sources of human outbreaks [22] and some studies have hypothesized that a difference in cytokine response of human PBMC to cattle-associated genotype could explain this fact [23]. However, in Poland, human outbreaks seem to be more frequently related to cattle [24]. In addition, MST 20 genotype has been detected in cattle from several countries all over the world (Spain [25], Hungary [26], the Netherlands [27] and the USA [28]) but also in humans endocarditis (heart valve), valvular prostheses and aneurysms [10]. MST20 has also been isolated from placentas from aborted women in France [11]. These elements suggest that *C. burnetii* shedding in cattle is also a significant source of human contamination.

In our study, the viability of *C. burnetii* bacteria is not proven, but the presence of DNA in placental tissue from aborted dairy cows should encourage further studies on its implication in dairy cows abortion in Algeria. In this country, this is a major issue, because livestock farming represents a source of financial income to a significant part of the population, with approximately two million cattle, 27.8 million sheep, 5.1 million goats and 0.35 million camels [29]. We conclude, therefore, that collaboration between the Ministry of Health and the Directorate of Veterinary Services in this country is crucial to better know *C. burnetii* infection prevalence in cattle and its consequences in terms of reproductive disorders and risks for human health.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2018.06.001.

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