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

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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],

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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].

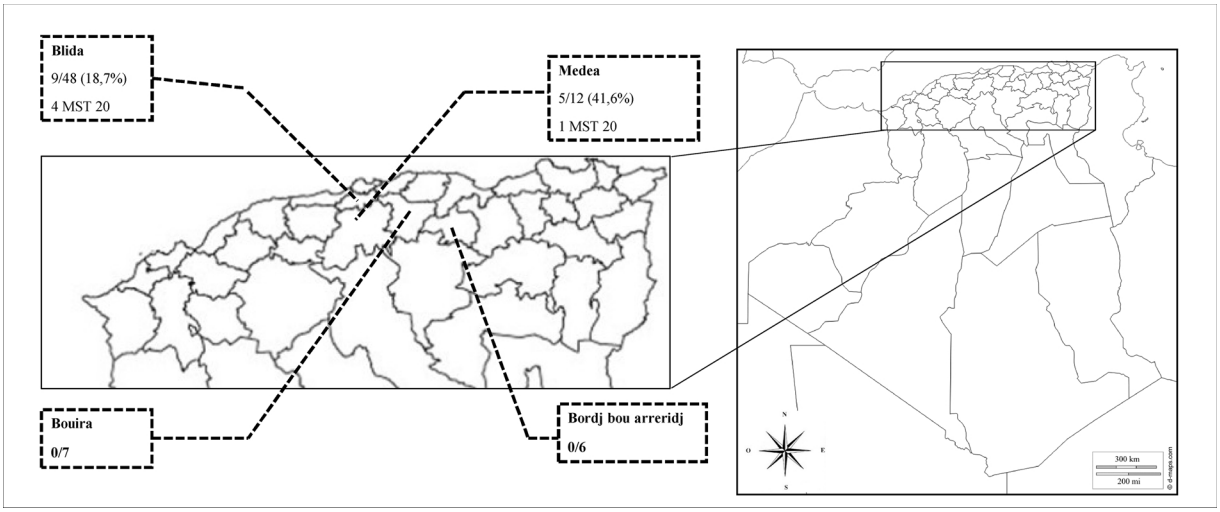

Fig. 1. Map of Algeria showing the geographical distribution of positive samples and according found MST genotypes of *C. burnetii*.