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Loss of TSS1 in hypervirulent *Coxiella burnetii* 175, the causative agent of Q fever in French Guiana

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ABSTRACT

In French Guiana, the unique *Coxiella burnetii* circulating genotype 17 causes 24% of community-acquired pneumonia, the highest prevalence ever described. To explain this unusual virulence, we performed a comparative genomic analysis of strain Cb175, which was isolated from a patient from French Guiana. Cb175 has a greater number of mutations in genes involved in metabolism compared with the Nine Mile I strain. We found a 6105 bp fragment missing in Cb175, which corresponds to the Type 1 secretion systems (T1SS) hlyCABD operon region. This deletion was detected by a specific qPCR in the 8 other strains available from this territory an in none of 298 *C.burnetii* strains from other areas and other genotypes (8/8 vs 0/298, Fisher's exact test, $p < 0.0000001$). Loss of genes implicated in secretion systems has been observed in other epidemic bacterial strains. Thus, the virulence of Cb175 may be linked to this genome reduction.

1. Introduction

Coxiella burnetii is an obligate intracellular bacterium that belongs to the Gamma-Proteobacteria family [1] and causes Q fever, a worldwide occurring zoonosis [2]. In the acute form of the disease, clinical presentation ranges from asymptomatic seroconversion to various clinical conditions, such as flu-like symptoms, hepatitis or pneumonia. The clinical presentation and severity of Q fever can vary depending on the strain of *C. burnetii* involved [3]. In metropolitan France, where Q fever is endemic, the annual incidence of acute infection is estimated to be 2.5 per 100,000 inhabitants [4], and fever and transaminitis are the most common clinical symptoms [5]. The epidemiology, clinical features and serological responses of Q fever reported in French Guiana are different from what has been described throughout the rest of the world [6].

Since its first report in 1955 [7], the incidence of acute Q fever in Cayenne increased to 37 per 100,000 inhabitants in 1996 [8]. That year, 3 patients were admitted to the intensive care unit in Cayenne Hospital for Q fever pneumonia, and one patient died as a result of distress respiratory syndrome [6]. Subsequently, the rate of incidence has continued to increase and peaked at 150 per 100,000 inhabitants in 2005 [9]. Q fever currently represents 24% of cases of community-acquired pneumonia in Cayenne [10]. Patients with Q fever pneumonia in Cayenne exhibit a more severe initial presentation with significantly more frequent chills, night sweats, headache and arthromyalgia than patients with other etiologies of pneumonia. These patients also have a more marked inflammatory response with higher CRP levels, but lower leukocyte counts [10]. Additionally, Q fever patients from Cayenne have a higher prevalence of fever (97%) and pneumonia (83%) than patients from metropolitan France (81% and 8%, respectively) [7]. Regarding the serological response, Guianan Q fever patients exhibit higher levels of phase I antibodies in the acute form of the disease [7].

In 2012, five *C. burnetii* isolates obtained from samples of five patients from Cayenne were cultured for the first time [10]. Genotypic analysis of these strains revealed that a single clone (MST17) circulates in Cayenne and is related to genotypes that harbor the QpH1 plasmid. This clone is epidemic and has been present in Cayenne since at least 2000 [11], the year that this first isolate was sampled.

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We performed a comparative genomic analysis of one of these strains, Cb175, to investigate why this genotype causes more virulent acute forms than other strains that have been previously described.

2. Methods

2.1. Strain culture, basic genomic data and sequencing

The Cb175 strain was isolated in May 2012 from the cardiac valve of a 60-year-old patient living in French Guiana who underwent an operation for blood-culture negative endocarditis with cardiac dysfunction [11]. This strain and all other *C. burnetii* strains from patient samples were cultured at 35 °C on L929 cells using MEM (GIBCO, Invitrogen, Cergy-Pontoise, France) supplemented with 4% FBS (GIBCO) and 1% L-glutamine (GIBCO). Monolayers of cells and the supernatants from three 175 cm^2 flasks were harvested and incubated with 1% trypsin (GIBCO) for 1 h at 37 °C. The released bacteria were purified from L929 cell debris on a discontinuous Gastrografine (Schering, Lys-Lez-Lannoy, France) gradient (45%, 36% and 28%) and ultracentrifuged at 5000 Tars/min for 1 h 10 min. The genome of the culture-positive strain Cb175 was subjected to paired-end SOLiD sequencing (run accession ERR845240). DNA was extracted using a QIAamp® DNA Mini Kit protocol by performing two subsequent elutions in a total volume of 30 μl. The paired-end library was constructed from 1 μg purified genomic DNA (58 ng/μl) after quantification using the Quant-iT™ PicoGreen® dsDNA Assay Kit from Life Technologies. Sequencing was carried out to 50 × 35 bp using SOLiD™ V4 chemistry on one full slide that was associated with 95 other projects on an Applied Biosystems SOLiD4 machine. All 96 genomic DNA samples were barcoded with the module 1–96 barcodes provided by Life Technologies and were fragmented on a Covaris device. The concentration of the library was measured on the Qbit fluorometer as 20.3 nmol/l. Libraries were pooled in equimolar ratios and size-selected on the E-Gel iBase system at 240–270 bp. PCR was performed on the EZ beads automated Emulsifier, Amplifier and Enricher E80 using the full-scale template bead preparation kit according to the protocol provided by Life Technologies. A total of 708 million P2-positive beads were loaded onto the flow cell. The output paired read length was 85 bp (50 × 35 bp). Of the total of 39.8 Gb for the full slide, the *C. burnetii* Cb175 project yielded 7,529,649 barcoded paired reads, which add up to 640 Mbp. Among a total of 23,149,703 reads obtained for four *C. burnetii* strains sequenced in the same SOLiD slide, 21.7% were associated with Z3055, 32.5% with Cb175, 27% with Cb51 and 18.8% with the HenzerlingS strain.

2.2. Genotyping and finishing

Genotyping. Multi-spacer sequence typing (MST) was performed to define the genotype of the strain. This consisted of PCR and sequencing of the following 10 spacers of the *C. burnetii* genome: Cox2, Cox 5, Cox 6, Cox 18, Cox 20, Cox 22, Cox 37, Cox 51, Cox 56, Cox 57 and Cox 61 [3]. Subsequently, these results were confirmed by in silico genotyping. Using a web-based MST database (http://ifr48.timone.univ-mrs.fr/MSTCoxiella/mst), we aligned each Cox sequence with the Cb175 genome.

Finishing. The reads obtained were mapped against the reference Nine Mile I (NC_002971.3) genome using CLC Genomics Workbench 6.0 (copyright CLC bio) using the parameters length fraction = 0.5 and similarity fraction = 0.8, which allowed us to obtain a consensus of 1,992,640 bp with an average coverage of 154 and fraction of reference covered of 100%.

Then, gap finishing was performed by multiple rounds of PCR and sequencing using BigDye terminator chemistry on the ABI3730 sequencing machine (Applied Biosciences) to complete the consensus sequence. Standard PCR was conducted in a total volume of 20 μl containing 0.02 U Phusion High-Fidelity DNA Polymerase (Finnzyme, Thermo Scientific), 200 μM dNTPs, 1× reaction buffer, 0.5 μM of each amplification primer, and 0.5 μl template DNA. Finally, we performed a mismapping analysis in order discard regions incorrectly placed. During finishing step, a 6105 bp deletion region, compared to the reference genome of NM I, was detected.

2.3. Determination of missing region in other Coxiella burnetii strains from Guiana

A qPCR system was designed to target the 6105 bp deletion by choosing the sequences F-GTGACGTTTATGGTTACTCATG and R-CTCCGATGCGGATAAATCCTA on each side of the deleted region as the primers and 6-FAM-AATCCGCCGGAACAGTCGTCAAC-TAMRA as the probe targeting the 23 bp region flanking the deletion (Fig. 1). Using this strategy, a positive PCR product was only obtained in strains of *C. burnetii* that had this deleted region. Primer and probe specificity were verified in silico by a BLAST search of GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) and by qPCR of a panel of 15 *C. burnetii* strains, including 5 strains of *C. burnetii* MST17 and 10 other genotypes of *C. burnetii* (Table 1). Then, a larger sample size was tested that included five DNA samples from 5 patients with Q fever from Cayenne [11], 1 DNA sample from the feces of a three-toed sloth, 2 DNA samples from ticks collected from a three-toed sloth from Cayenne [12] and 298 other DNA samples from patients diagnosed at our center with genotypes other than MST17.

2.4. Investigation of the genomic sequence corresponding to the missing region in the Guiana strain in other Coxiella burnetii genomes

The missing region in Cb175 was searched for in the following other *C. burnetii* strains sequenced by our laboratory: 21 strains sequenced using Illumina MiSeq technology, 13 strains sequenced using SOLiD and one strain sequenced using the 454 shotgun method. Reads obtained by sequencing were mapped against the missing region using stringent parameters (length fraction = 0.95 and similarity fraction = 0.95). An identical method was used for strain Cb109 [13], which has been sequenced in our laboratory, but does not have a completely annotated genome. Additionally, a BLASTN analysis [14] of the missing region was performed against the *C. burnetii* genomes available in GenBank, including the Dugway 5J108-111 [15], CbuG_Q212 [15,16], CbuK_Q154 [15], RSA 331, Nine Mile phase I (NMI) [15,16], Z3055 [17], Q321, MSU Goat Q177, Cb_C2 [18], Cb_B1 [18], EV-Cb_BK10 [18], Cb_O184 [18], EV-Cb_C13 [18] and Cb_B18 [18] strains.

2.5. Genetic variability

The Cb175 genomic sequence was compared with the 6 complete genomes available in GenBank: Dugway 5J108-111, CbuG_Q212, CbuK_Q154, RSA 331, Nine Mile, and Z3055 (for which we have submitted also plasmid sequence LN827801). The GenMark software [19] was used to predict and translate the coding regions (CDs). The CDs of each genome were concatenated and dereplicated by clustering using USEARCH [20] at an identity of 0.9 to reduce the size of dataset. TBLASTN [21] of the translated sequences was performed against each genome of the dataset and the query bit score for each genome was tabulated. The query bit score was divided by the maximum bit score for all genomes to calculate the blast score ratio [22,23], which could range from 1.0 (exact peptide