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Présentée par
Madame Carole ELDIN

COXIELLA BURNETII : DE LA CULTURE AUX MANIFESTATIONS CLINIQUES

Soutenue le 7 février 2017 devant le jury

Mr Le Professeur Jean-Louis MEGE	**Président du Jury**
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AVANT PROPOS :

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

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SOMMAIRE

RESUME:

Coxiella burnetii (C. burnetii) est l'agent de la fièvre Q, zoonose décrite pour la première fois en 1937. Nous avons tout d'abord synthétisé l'état des connaissances actuelles sur cet agent infectieux par une revue de la littérature. Puis nous avons réalisé un travail sur cette bactérie autour de trois thèmes : (1) Les conditions de culture de la bactérie (2) Les déterminants de la virulence (3) Le diagnostic et le traitement de l'infection *à C. burnetii* chez l'homme.

C. burnetii étant une bactérie intracellulaire, son isolement au laboratoire repose sur une culture sur cellules. Récemment, un milieu nommé ACCM 2 (Acidified Cysteine Citrate Medium 2) a été développé, et permet désormais la culture axénique de cette bactérie en atmosphère microaérophile (2,5% d'Oxygène). Nous avons voulu tester si l'ajout de molécules antioxydantes dans le milieu de culture pouvait limiter le stress oxydatif et ainsi permettre une culture en milieu aérobie. Nous avons donc ajouté de l'acide urique dans le milieu ACCM2. Nous avons observé une croissance de *C. burnetii* incubée en conditions aérobies à J7 de culture dans le milieu ACCM2 enrichi en acide urique.

La virulence des infections causées par *C. burnetii* est variable d'une région à l'autre. A Cayenne, en Guyane Française, 24% des pneumopathies communautaires sont causées par *C. burnetii* et ces pneumopathies sont plus sévères. Nous avons participé à l'analyse du génome d'une souche isolée à Cayenne. Ce travail a mis en évidence une délétion de 6105 pb induisant une réduction de génome et intéressant le gène du système de sécrétion de type 1 (T1SS). Cette réduction de génome est probablement impliquée dans la plus grande virulence des souches isolées à Cayenne. En amont de ce travail nous avons participé à une revue de la littérature sur l'apport de la génomique dans l'étude de *C. burnetii*. Enfin, nous avons testé la sensibilité aux antibiotiques de 6 souches isolées à partir de patients vivant à Cayenne. Ces souches étaient

toutes sensibles à la doxycycline mais résistantes aux macrolides, dont une résistante à la télithromycine, ce qui n'avait jamais été décrit auparavant chez *C. burnetii*.

Dans une troisième partie nous avons étudié les manifestations cliniques de l'infection à *C.burnetii*. Nous avons d'abord testé l'apport du PET scanner dans le diagnostic des infections à *C. burnetii*. 167 patients atteints d'infections à *C. burnetii* ont bénéficié d'un PET scanner entre 2009 et 2015. Le PET scanner a permis de changer le diagnostic initial en confirmant ou en découvrant un foyer infectieux pour 62 patients. Nous avons retrouvé une proportion élevée de fixations ostéo-articulaires (21) et ganglionnaires (27), ce qui nous a amené à proposer de nouvelles définitions pour ces entités cliniques. Dans une autre étude, nous avons étudié l'impact du traitement chirurgical chez les patients atteints d'infections vasculaires à *C. burnetii*. Une analyse rétrospective de 100 patients atteints d'infections vasculaires à *C. burnetii* entre 1986 et 2015 a montré que le traitement chirurgical était associé à une diminution de la mortalité à 2 ans et demi et permettait également une meilleure évolution sérologique.

Nos travaux associés à la synthèse des connaissances actuelles sur cet agent infectieux a permis de constater qu'un certain nombre de paradigmes avaient changé concernant cette bactérie. Premièrement *C. burnetii* est désormais cultivable de façon axénique, ce qui facilitera notamment les techniques de transformation génétique. Deuxièmement, la virulence de la bactérie est liée à la fois à des facteurs génétiques, reflétés par les différents génotypes décrits, ainsi qu'à des facteurs liés à l'hôte. Troisièmement, la dichotomie entre fièvre Q « aigue » et « chronique » sème une forme de confusion, car elle et ne correspond plus à la multiplicité des localisations, pronostics et traitements de l'infection à *C. burnetii.*

Mots clés : *Coxiella burnetii*, culture, virulence, milieu axénique, PET scanner, infections vasculaires

ABSTRACT

Coxiella burnetii (*C. burnetii*) is the agent of Q fever, a zoonosis first described in 1937 . First we performed a review of the literature about this bacterium. Then, our work on this bacterium was based on three objectives: (1) Optimizing the culture of *C. burnetii*. (2) Studying the virulence determinants through the analysis of hypervirulent strains. (3) Improving the diagnosis and treatment of *C. burnetii* infections in humans. Finally, we synthesized all the current knowledge about this microorganism in the literature.

Being an intracellular bacterium, *C. burnetii* is routinely cultivated on cells. Recently, a medium called ACCM 2 (Acidified Cysteine Citrate Medium 2) has been developed and allows the axenic culture of this bacterium in a microaerophilic atmosphere (2.5% oxygen). Thus, we tested if the addition of antioxydant molecules in the medium could allow aerobic culture by limiting oxidative stress. We added uric acid in ACCM2. We observed a growth of *C. burnetii* incubated under aerobic conditions at day 7 of culture in ACCM2 medium enriched with uric acid.

The virulence of *C. burnetii* varies from one region to another. In Cayenne, French Guiana, 24% of community acquired pneumonia are caused by *C. burnetii* and these pneumonia are more severe. We took part in the analysis of the genome of a strain isolated in Cayenne. The genome of this strain revealed a deletion of 6105 bp, inducing a genome reduction and located in the type 1 secretion system (T1SS). This genome reduction is probably implicated in the greater virulence of strains isolated in Cayenne. Before this work we also participated in a review of the literature on the contribution of new genomic tools in the study of *C. burnetii*. Finally, we determined the antibiotic susceptibility of 6 strains isolated from patients living in Cayenne. These strains were all doxycycline-sensitive but resistant to macrolides, including one strain resistant to telithromycin, which was described for the first time.

In a third part, we focused on the clinical manifestations of *C. burnetii* infection. We

studied the contribution of 18F-FDG PET/CT in the diagnosis of *C. burnetii* infections. 167 patients with *C. burnetii* infections who underwent 18F-FDG PET/CT between 2009 and 2015 were included. The 18F-FDG PET/CT changed the initial diagnosis by confirming or discovering an infectious focus for 62 patients. We found a high proportion of osteo-articular (21) and lymph node (27) localizations, which led us to propose new definitions for these clinical entities. In another study, we investigated the impact of surgical treatment in patients with *C. burnetii* vascular infections. Our retrospective analysis of 100 patients with *C. burnetii* vascular infections between 1986 and 2015 showed that surgical treatment was associated with a lower mortality at 2.5 years of follow-up, and with a better serological outcome.

Our work, combined with an ehxhaustive review of the literature, revealed that several paradigms have changed in the conception of this bacterium. First *C. burnetii* is now cultivable in an axenic way, allowing genetic transformation. Then, the virulence of the bacterium is associated with genetic characteristics reflected by different genotypes and with host susceptibilities. Finally, the old dichotomy between "acute" and "chronic" Q is obsolete because it does not reflect the multiplicity of localizations, prognoses and treatments of *C. burnetii* infection.

Keywords : Coxiella burnetii, culture, virulence, axenic medium, 18F-FDG PET/CT, vascular infections

INTRODUCTION

C. burnetii a été décrite pour la première fois en 1937, simultanément par Burnet et Freeman à partir de tissus d'animaux infectés provenant d'une épidémie australienne et par Cox et Davis à partir de tiques du Montana (1, 2). Dès 1938, Cox décrivit ses tentatives infructueuses de culture axénique de *C. burnetii*, avant de réussir à la cultiver sur des œufs embryonnés et sur des modèles animaux tels que la souris ou le cochon d'inde (3). *C. burnetii* a ainsi été définie à partir de ce travail comme une bactérie intracellulaire stricte. Au sein de la cellule hôte, il a depuis été montré que la bactérie se réplique dans une vacuole intracellulaire aux caractéristiques physico chimiques extrêmes, (pH acide, hydrolases, peptides cationiques). Pour améliorer la prise en charge des patients présentant une infection *C. burnetii*, il est nécesaire de mieux comprendre les facteurs de virulence de celle-ci. Les méthodes de génomique actuelles sont un des outils pour cela, mais en amont il est nécessaire de pouvoir cultiver plus facilement cette bactérie.

La culture sur cellules reste jusqu'à ce jour la principale méthode permettant d'isoler *C. burnetii* à partir d'échantillons cliniques. Récemment, un milieu de culture axénique pour *C. burnetii*, nommé ACCM2 pour Acidified Cystein Citrate Medium a été développé. Ce milieu permet une croissance de 4 à 5 log en 7 jours pour la souche Nine Mile de phase I et II et des microcolonies sont observées en milieu solide. Les principales caractéristiques de ce milieu sont les suivantes: un pH acide à 4,5 obtenu grâce à un tampon citraté, une richesse en cystéine, la présence du milieu de culture cellulaire RPMI, la présence de néopeptone, de casamino acids et de Méthyl Béta cyclodextrine. L'un des facteurs les plus critiques pour la croissance de *C. burnetii* identifié par les auteurs est le maintien de conditions atmosphériques microaérophiles à 2.5% d'oxygène. Ces conditions sont obtenues à l'aide d'une étuve à pression d'oxygène contrôlée. Récemment, une équipe allemande est parvenue à isoler C. burnetii à partir d'échantillon cliniques grâce à ce milieu de culture (4).

Pour comprendre les déterminants de la virulence, les avancées de la génomique sont un outil puissant. Elles ont permis de séquencer plusieurs souches de *C. burnetii* depuis 2003, date de publication du premier génome complet de la souche RSA493 (5). Toutes les souches séquencées jusqu'à présent ont montré une homogénéité génomique forte. Toutefois, des techniques de génotypage ont été développées telles que le Multispacer Sequence Typing, qui représente un outil majeur dans la description de la diversité des souches de *C. burnetii*. Une récente étude a ainsi montré que toutes les souches isolées à Cayenne en Guyane Française, présentaient le même génotype, dénommé MST 17 (6). Or, l'épidémiologie de l'infection à *C. burnetii* à Cayenne est unique. Dans cette ville, C. burnetii cause 24% des pneumopathies communautaires, ce qui représente la prévalence la plus élevée au monde (7).

En dehors des pneumopathies qui sont une des présentations cliniques de la primo infection à *C. burnetii,* la bactérie peut donner lieu à plusieurs autres tableaux cliniques, qui dépendent à la fois de la souche impliquée et des caractéristiques de l'hôte (immunodépression, valvulopathie, grossesse). La localisation du foyer infectieux est difficile dans ces infections car les modifications anatomiques sont parfois très ténues. Par exemple, une végétation est visible dans seulement 30% des endocardites à *C. burnetii* (8), et les infections d'anévrysmes vasculaires peuvent être peu symptomatiques pendant des années avant de se révéler par une complication sévère telle qu'une fistulisation digestive ou une rupture (9). Ainsi, les infections vasculaires sont à ce jour la localisation au pronostic le plus défavorable. Une fois diagnostiquées, le traitement de ces infections repose sur une bithérapie par doxycyline et plaquenil, et une étude rétrospective a montré que la chirurgie semblait associée à un meilleur pronostic (9).

Le TEP scanner est un outil d'imagerie qui consiste à injecter un traceur faiblement radioactif, le 18 F fluoro-déoxyglucose, qui va se fixer dans les tissus cancéreux ou inflammatoires. Ceci en fait donc un examen d'imagerie fonctionnelle, dont les indications ne

cessent de s'étendre notamment dans le domaine des maladies infectieuses (fièvres prolongées, endocardites) (10, 11).

Dans une première partie de ce travail, nous avons réalisé une revue de la littérature sur *C. burnetii*.

Dans une deuxième partie nous avons testé l'ajout d'une molécule antioxydante, l'acide urique, dans le milieu ACCM2. Ceci avait pour but de cultiver *C. burnetii* en atmosphère aérobie, ce qui faciliterait la culture de routine de ce pathogène.

Dans une troisième partie, après avoir réalisé une revue de la littérature sur l'apport de la génomique dans l'étude de *C. burnetii,* nous avons participé à l'analyse du génome de la souche Cb 175 isolée à Cayenne en Guyane Française. Nous avons également déterminé la sensibilité aux antibiotiques de 6 souches isolées à partir de patients vivant ou ayant vécu à Cayenne.

Enfin, nous avons décrit rétrospectivement l'apport du TEP scanner dans le diagnostic des localisations des infections à *C. burnetii.* Nous avons également réalisé une étude rétrospective évaluant l'impact du traitement chirurgical sur le pronostic des infections vasculaires à *C. burnetii.*

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Partie I : Revue de la littérature

AVANT-PROPOS

Coxiella burnetii est l'agent de la fièvre Q, ou « Query Fever », une zoonose décrite pour la première fois en Australie en 1937. Depuis cette première description, la connaissance de ce pathogène et des manifestations cliniques qu'il peut provoquer a considérablement évolué. Nous avons réalisé une revue exhaustive de la littérature de ces 20 dernières années concernant *C. burnetii*. Nous avons constaté que plusieurs changements de paradigmes avaient eu lieu dans la conception traditionnelle que l'on avait de cette bactérie.

Tout d'abord, *C. burnetii* a longtemps été considérée comme une bactérie intracellulaire stricte. Or, l'élaboration récente d'un milieu de culture axénique a totalement révolutionné cette conception. Ce milieu dénommé ACCM2 permet une croissance de 5 log en 7 jours, et il a été utilisé récemment pour l'isolement de *C. burnetii* à partir de prélèvements cliniques (1, 2). La culture axénique de *C. burnetii* ouvre également la voie à des méthodes de transformation génétique autrefois beaucoup plus fastidieuses, ce qui permettra probablement de mieux comprendre les mécanismes de virulence de ce pathogène.

C. burnetii est pathogène chez la plupart des espèces, des arthropodes aux humains et la bactérie a été détectée sur les cinq continents. Cependant, l'épidémiologie de la maladie est éminemment variable d'une zone géographique à une autre. En effet, la bactérie peut être à l'origine d'épidémies massives comme cela a été le cas aux Pays-bas (3), tandis qu'elle peut persister à l'état endémique ou hyperendémique dans d'autres pays. Le développement récent de plusieurs méthodes de génotypage permet désormais d'avoir une idée de la diversité des souches en fonction des zones géographiques (4). Ceci permet également de retracer l'origine des souches impliquées dans les épidémies grâce à des analyses phylogénétiques. Par ailleurs, les déterminants génétiques de la virulence de *C. burnetii* sont désormais mieux connus, grâce au séquençage de plusieurs souches, complété par des analyses de génomique comparative (5–7). Ainsi, la présentation clinique de l'infection à *C. burnetii* dépend à la fois de la virulence de

la souche infectante (déterminée génétiquement) et des facteurs de risque spécifiques de l'hôte (sexe, âge, valvulopathie, anevrysme ou prothèse vasculaire, grossesse).

Sur le plan des manifestations cliniques, l'une des avancées majeures de ces dernières années est la rupture avec l'ancienne dichotomie entre fièvre Q « aigue » et « chronique ». En effet cette classification est simplificatrice à plusieurs égards, notamment parce qu'elle reflète mal l'éventail des manifestations cliniques et des localisations de l'infection à *C. burnetii*. En outre, la notion de fièvre Q chronique repose sur une définition initiale sérologique, qui est la présence d'immunoglobulines de phase I à des taux élevés. Pourtant, cette définition sérologique a été mise en défaut à plusieurs reprises, notamment chez les patients présentant des primo-infections à *C. burnetii* à Cayenne, en Guyane Française (8). En effet, ceux-ci présentent des taux d'IgG de phase I très élevées, sans pour autant avoir des infections cliniquement persistantes. Par ailleurs, l'étiquette abstraite de « fièvre Q chronique » empêche parfois la poursuite d'investigations cliniques qui pourraient permettre de localiser le ou les foyers infectieux. En effet, il est impossible de considérer qu'une infection chronique puisse persister sans foyer infectieux identifié. Ce changement de paradigme dans la conception des infections causées par *C. burnetii* devrait permettre un meilleur diagnostic et une meilleure prise en charge de la primo infection et des infections persistantes localisées à *C. burnetii.*

Review : From Q fever to *C. burnetii* infection : a change of paradigm

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REVIEW

From Q Fever to *Coxiella burnetii* Infection: a Paradigm Change

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SUMMARY *Coxiella burnetii* is the agent of Q fever, or "query fever," a zoonosis first described in Australia in 1937. Since this first description, knowledge about this pathogen and its associated infections has increased dramatically. We review here all the progress made over the last 20 years on this topic. *C. burnetii* is classically a strict intracellular, Gram-negative bacterium. However, a major step in the characterization of this pathogen was achieved by the establishment of its axenic culture. *C. burnetii* infects a wide range of animal, from arthropods to humans. The genetic determinants of virulence are now better known, thanks to the achievement of determining the genome sequences of several strains of this species and comparative genomic analyses. Q fever can be found worldwide, but the epidemiological features of this disease vary according to the geographic area considered, including situations where it is endemic or hyperendemic, and the occurrence of large epidemic outbreaks. In recent years, a major breakthrough in the understanding of the natural history of human infection with *C. burnetii* was the breaking of the old dichotomy between "acute" and "chronic" Q fever. The clinical presentation of *C. burnetii* infection depends on both the virulence of the infecting *C. burnetii* strain and specific risks factors in the infected patient. Moreover, no persistent infection can exist without a focus of infection. This paradigm change should allow better diagnosis and management of primary infection and long-term complications in patients with *C. burnetii* infection.

KEYWORDS *Coxiella burnetii*, diagnosis, Q fever, treatment, epidemiology, genomics

INTRODUCTION

AQ: A

Q fever has long been considered a rare and regionally restricted disease. In recent years, spectacular advances have been made in the knowledge of this disease and its causative agent, *Coxiella burnetii*. First, the worldwide role of *Coxiella burnetii* as a cause of endocarditis has been recognized in most countries performing systematic serology. Moreover, the classification of *C. burnetii* by the CDC as a potential bioterrorism agent resulted in the disease becoming reportable in many countries, such as in the United States, which revealed that the disease is more common than previously thought. Third, the recent war in the Middle East (1) and research in the tropics (2, 3) have shown that Q fever may be a very common cause of fever in the intertropical area. Finally, a very large outbreak in the Netherlands has shown that this disease could become a major public health problem (4).

Furthermore, knowledge about *Coxiella burnetii* has evolved, with the sequencing of multiple genomes of bacterial strains and their culture in axenic medium. This breakthrough enables genetic transformation and opens a new era. Moreover, redefining the clinical forms of Q fever is necessary, because of a lack of consensus on the distinction between acute Q fever and chronic Q fever (5). This redefinition, by more precisely qualifying the different clinical forms of the disease, will improve the exchange of medical and scientific knowledge about the disease throughout the world.

SEARCH STRATEGY

We searched in Medline and Google scholar for references with no language restriction and no restriction of publication status, using the key words "*Coxiella burnetii*" OR "Q fever" AND the other key words "diagnosis, treatment, epidemiology, human, animal, pathophysiology, chronic, acute." We applied no time restriction. This search yielded 12,887 references. We removed duplicates and assessed the remaining

AQ: B references for eligibility. Finally, 687 references were included in the qualitative analysis. Data were collected and extracted from the selected studies and synthesized in the appropriate section.

MICROBIOLOGY

The Bacterium

C. burnetii has a cell wall similar to that of Gram-negative bacteria. However, this small coccobacillus (0.2 to 0.4 μm wide and 0.4 to 1 μm long) is not stainable with the Gram technique. The Gimenez method is used to stain *C. burnetii* isolated in culture or directly in clinical samples (6). The estimated doubling time of the bacterium is between 20 and 45 h in *in vitro* cell culture (7). It is an intracellular pathogen, replicating in eukaryotic cells. Its vacuole of replication progressively acquires phagolysosome-like

AQ: C characteristics, such as an acidic pH, acid hydrolysates, and cationic peptides (8). The bacterium actively participates in the genesis of this intracellular vacuole and has several strategies for adaptation to this exceptionally stressful environment. First, *C. burnetii* encodes an important number of basic proteins that are probably involved in the buffering of the acidic environment of the phagolysosome-like vacuole (9). Also, four sodium-proton exchangers and transporters for osmoprotectants are found in the *C. burnetii* genome, allowing this bacterium to confront osmotic and oxidative stresses.

Two forms of this microorganism can be observed, corresponding to a biphasic developmental cycle. The large-cell variant (LCV) of the bacterium is an exponentially replicating form, whereas the small-cell variant (SCV) is a stationary nonreplicating form (10). SCVs are small rods (0.2 to 0.5 μm long) characterized by condensed chromatin, a thick envelope, and an unusual internal membrane system. LCVs have a larger size (>0.5 μm), a dispersed chromatin, and an envelope similar to that of classical Gram-negative bacteria. SCVs are typical of the stationary phase. They are observed after prolonged culture (21 days) in Vero cells and in axenic acidified cysteine citrate medium 2 (ACCM2) (11). SCVs are stable in the environment and are highly resistant to osmotic, mechanical, chemical, heat, and desiccation stresses. These properties led to the adoption of a high temperature (71.7°C) for pasteurization in the 1950s (12). The transcriptome analysis of the SCV has revealed upregulated genes involved in the oxidative stress response, cell wall remodeling, and arginine acquisition (10). Also, SCVs show an unusually high number of cross-links in their peptidoglycan, which probably are involved in their exceptional environmental resistance (10). They can survive for 7 to 10 months on wool at ambient temperature, for more than 1 month on fresh meat, and for more than 40 months in milk (7). Although the SCVs are destroyed by 2% formaldehyde, they have been isolated from tissues stored in formaldehyde for 4 to 5 months (7). The high virulence of *C. burnetii*, the possibility of its aerosolization, and its environmental stability and have led the U.S. Centers for Disease Control and Prevention to classify this bacterium as a category B biological threat agent. A bioterrorism attack with this pathogen, although not associated with the high death rates observed for class A agents, could cause significant disability and possibly long-term consequences due to persistent infection in the population.

Antibiotic Susceptibility and the Role of pH

Methods for AST. For years, the antibiotic susceptibility testing (AST) of *C. burnetii* was difficult, owing to the obligate intracellular lifestyle of this bacterium. Antibiotic activity was evaluated first in animal models, then in embryonated-egg models and finally in cell culture systems. Inoculation in guinea pigs was used for susceptibility testing of streptomycin in early studies (13). The embryonated-egg method involved the ability of the tested antibiotic to prolong the survival time of the embryo in eggs infected with *C. burnetii*. This method was used in old studies to test the activities of streptomycin, chloramphenicol, oxytetracycline, and aureomycin against *C. burnetii* (14).

Cell culture systems were then implemented and remain the reference method for *C. burnetii* AST. In 1987, Yeaman et al. used *C. burnetii*-infected L929 fibroblast cells and

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compared the percentage of infected cells (after Gimenez staining of bacteria) in antibiotic-treated cultures to that in drug-free controls (15). The more convenient shell vial assay was then developed, using specific antibodies and the immunofluorescence assay for detection of intracellular *C. burnetii* (16–18). In 2003, Brennan and Samuel developed a variant of the shell vial assay by using quantitative PCR (qPCR) for determination of *C. burnetii* intracellular counts (19). This method was found to be more repeatable and likely more sensitive than the indirect immunofluorescence assay (IFA) (19). It was then applied in another study from our team, which confirmed its excellent reproducibility for MIC determination (20). In recent years, both the IFA and qPCR tests targeting *com1* or *apoB* have been used for *C. burnetii* AST in various eukaryotic cell lines (21–24). We recently developed a new method using flow cytometry and specific immunofluorescent probes. This technique allows a very sensitive counting of *C. burnetii* cells because of specific morphological characteristics (25).

AQ: D

Main susceptibility features. Doxycycline is the most effective drug against *C. burnetii*, with MICs of <2 mg/liter in most reports (21, 23, 25). However, strains with acquired resistance to doxycycline have been described and represent a worrisome situation. The first resistant strain was isolated from a patient who died from *C. burnetii* endocarditis. The doxycycline MIC was 8 mg/liter, as determined using the shell vial assay and qPCR (26). In the same study, Rolain et al. found a correlation between the ratio of serum concentration to MIC for doxycycline and the rate of decline of anti-*C. burnetii* antibody titers in patients with *C. burnetii* endocarditis. For 16 *C. burnetii* strains isolated from cardiac valves removed from endocarditis patients, a ratio of serum concentration to MIC of >1 correlated with a rapid decline in specific antibody titers. A ratio between 0.5 and 1 was associated with a slower reduction in antibody titers. The only patient who died from endocarditis had a ratio of <0.5 (26). The whole genome of the *C. burnetii* strain infecting that patient (Cb109) was determined, but no specific sequence could be correlated with doxycycline resistance (27). Since then, two other isolates have been found to be resistant to doxycycline, including one goat isolate and another human isolate from a patient with acute Q fever (28).

In early studies, the fluoroquinolones were found to be one of the most effective agents in eliminating *C. burnetii* from L929 cells (15, 29). For that reason, in 1989 it was proposed to combine doxycycline with a fluoroquinolone to treat persistent forms of *C. burnetii* infection (30). Fluoroquinolones are also recommended for treatment of acute meningitis caused by *C. burnetii* because of the good cerebrospinal fluid penetration of these drugs (31). Pefloxacin- or ciprofloxacin-resistant strains of *C. burnetii* have been selected *in vitro* by Spyridaki et al. and Musso et al., with MICs up to 64 mg/liter (32, 33). These authors identified point mutations in the *gyrA* gene that could allow PCR-restriction fragment length polymorphism (PCR-RFLP) detection of these resistant strains (32). However, to date, clinical isolates of *C. burnetii* remain susceptible to levofloxacin, moxifloxacin, and to a lesser extent ciprofloxacin (12, 13, 16, 20).

Erythromycin was proposed as an empirical treatment for *C. burnetii* pneumonia. However, in 1991, Raoult et al. found that 6 of 13 clinical isolates of *C. burnetii* were resistant to this antibiotic (16), and such resistance was more recently observed in 6 isolates from Cayenne, French Guiana (11, 20). Conversely, clarithromycin was found to be active, with MICs between 2 and 4 mg/liter (12, 13). For azithromycin, higher MICs, up to 8 mg/liter, have been observed (16, 20). Telithromycin was considered active against *C. burnetii*, with MICs between 0.5 and 2 mg/liter for 13 clinical isolates (28). However, we recently isolated a strain from French Guiana which was resistant to this antibiotic (25).

AQ: E

No resistance to sulfamethoxazole-trimethoprim has been reported to date, suggesting that this agent is useful during pregnancy. Anecdotal reports observed susceptibility to tigecycline and linezolid and proposed them as alternative agents (13, 18). Unsworth et al. recently reported susceptibility of *C. burnetii* to antimicrobial peptides (24). Other nonantibiotic agents have been reported to display *in vitro* activity against *C. burnetii*. Lovastatin and pentamidine can inhibit *C. burnetii* growth *in vitro* (683, 684).

Also, omeprazole is effective in reducing the size of *C. burnetii* intracellular vacuoles (34).

Role of pH in persistent infection. The antibiotic treatment of persistent *C. burnetii* infection has long been challenging, because no antibiotic has displayed a bactericidal effect. In 1990, it was demonstrated that the acidic environment of the phagolysosome-like vacuole where *C. burnetii* multiplies inhibited antibiotic activity. The acidification of the *C. burnetii* replication vacuole was stable over time for the three strains tested in an *in vitro* persistent cell infection model (35). For that reason, Raoult et al. combined alkalinizing agents with doxycycline and observed a restoration of the bactericidal effect of doxycycline (36). Among alkalinizing agents, chloroquine and amantadine were used, and doxycycline was the antibiotic for which restoration of the bactericidal activity was the highest. These results were subsequently confirmed clinically by comparing the outcomes of patients with *C. burnetii* endocarditis treated with the combination of either doxycycline plus a fluoroquinolone or doxycycline plus hydroxychloroquine. Patients who benefited from the latter combination had a shorter duration of treatment and less frequent relapses (37).

Recent Advances in Culture Techniques

For years, the strict intracellular nature of *C. burnetii* had been an experimental obstacle, with time-consuming culture methods. Our laboratory developed an empirical medium based on Vero cell extract (VCEM) that allows host cell-free cultivation of *C. burnetii* (38). However, adding fresh medium every 48 h was necessary to sustain *C. burnetii* growth, and to date no growth has been obtained on the solid agarose-VCEM (38).

Another axenic medium, called acidified cysteine citrate medium 2 (ACCM2), was developed. First, Omsland et al. in 2008 elaborated a complex *Coxiella* medium (CCM) composed of an acidic citrate buffer and a mixture of three complex nutrient sources (neopeptone, fetal bovine serum, and RPMI medium) (39). This composition was based on an *in silico* genomic analysis of metabolic deficiencies. The authors observed sustained metabolic activity of *C. burnetii* in this medium, which was measured with [^{35}S]Cys-Met incorporation (39). Other metabolic requirements were then analyzed using transcriptomic microarray, genomic reconstruction, and metabolite typing. This work led to the formulation of ACCM2, a medium with an acidic pH incubated in a 2.5% oxygen atmosphere. This medium allowed a substantial growth of about 3 logs of *C. burnetii* after 7 days of incubation. Microcolonies of *C. burnetii* were observed on solid agar plates (40). This medium was subsequently improved by adding methyl-β-cyclodextrin. An increased growth of 4 to 5 logs was obtained at day 7, and an easier isolation from animal tissue and genetic transformation was achieved with solid ACCM2 (41).

EPIDEMIOLOGICAL CHARACTERISTICS OF *C. BURNETII* INFECTION

Overview

Q fever cases have been reported almost everywhere they have been sought, except in New Zealand. The main reservoirs of *C. burnetii* are cattle, sheep, and goats. However, in recent years, an increasing number of animals have been reported to shed the bacterium, including domestic mammals, marine mammals, reptiles, ticks, and birds (42). Birth products contain the highest concentration of bacteria, but *C. burnetii* is also found in urine, feces, and milk of infected animals (7, 43, 44). Transmission to humans is most frequently due to inhalation of aerosolized bacteria that are spread in the environment by infected animals after delivery or abortion.

Because Q fever is a zoonosis, with only anecdotal reports of human-to-human transmission, the epidemiology of human infections always reflects the circulation of the bacterium in animal reservoirs. The prevalence of Q fever is highly variable from one country to another, due to epidemiological disparities and whether or not the disease is reportable. For example, Q fever became a reportable disease only in 1999 in the United States, which led to an increase of 250% in the number of human cases between

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2000 and 2004 due to better recognition of cases (45). Depending on the geographic area, endemic or outbreak situations are observed. In areas of endemicity, Q fever occurs as sporadic cases, usually after identifiable at-risk activities (farming, slaughterhouse work, or rural tourism). This is the predominant presentation in France, Spain, and the United States. In these countries some hyperendemic foci also can be identified, as in Martigues, a city in southeastern France, where the Q fever incidence rate reaches 34.5/100,000 inhabitants due to dissemination of spores by the local mistral wind from sheep herds breeding in the local plains (46). Small outbreaks (especially familial outbreaks) may occur after exposure to a common source, such as *C. burnetii*-infected parturient pets such as dogs or cats (47, 48). Also, an anecdotal outbreak was recently reported in the United States in five patients, due to intramuscular injection of fetal sheep cells by a German doctor practicing "live cell therapy," a xenotransplantation practice with no published evidence of efficacy (49).

Finally, large-scale outbreaks can occur at a country level, which happened in the Netherlands between 2007 and 2010, with more than 4000 reported cases. In the following section, we detail three of the most striking epidemiological profiles observed in recent years; the hyperendemicity situation in Africa, the major outbreak in the Netherlands, and the epidemic in Cayenne, French Guiana, associated with unique virulence and reservoir features.

The Different Epidemiological Profiles

Q fever in Africa. In 1955, Kaplan and Bertagna reported the first cases of Q fever in nine African countries, from Morocco to South Africa, suggesting that the infection was widespread in that continent (50). Seroprevalence studies then revealed the highest seropositivity rates in Mali, Burkina Faso, Nigeria, and Central African Republic, which are countries with the highest density of domestic ruminants (>100 per 100 inhabitants) (51). Q fever seroprevalence rates in humans varied from 1% in Chad (52) to 16% in Egypt (53). More recently, a seroprevalence study among blood donors in Namibia identified a 26% seropositivity rate (54). In an agropastoral region of Algeria, seroprevalence rates of 15%, with peaks up to 30% in villages where the disease is hyperendemic, have been observed (55). In Senegal, 24.5% of the population tested seropositive for *C. burnetii* in a rural village, and the bacterium was detected by PCR in 6 of 511 febrile patients (2, 56).

Because there are no easy diagnostic tools available in most African countries, the global impact of Q fever on public health has largely been underestimated. In a recent study, *C. burnetii* was found as the etiological agent in 5% of 109 severe pneumonia cases in Tanzania (57). In the same country, an investigation in a cohort of severely ill febrile patients found 26.2% zoonoses, among which 30% were Q fever (58). In Tunisia and Algeria, *C. burnetii* accounts for 1 to 3% of infective endocarditis (59). In Burkina Faso, Q fever is responsible for 5% of acute febrile illnesses (59). In Cameroon, 9% of community-acquired pneumonia in those aged >15 tested positive for *C. burnetii* (59). In that country, *C. burnetii* was the third most frequent agent of pneumonia, after *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* (60). These studies may be the tip of the iceberg, indicating widespread circulation of the bacterium in rural Africa.

Moreover, in most African countries, seroprevalence rates are elevated in domestic ruminants. Surveys in cattle showed rates ranging from 4% in Senegal to 55% in Nigeria (61, 62). In Egypt, a high rate of 33% seropositivity was observed in sheep herds (63). Goat seropositivity for *C. burnetii* ranged from 13% in Chad to 23% in Egypt. Also, Schelling et al. found 80% of camels with *C. burnetii* seropositivity (52), and a similar rate of 70% was found in Egypt (64), suggesting that these animals are significant reservoirs of the disease. In rural regions of most of these countries, human households are in close vicinity to domestic ruminants, making transmission easier than it is elsewhere. As a consequence, *C. burnetii* DNA was detected in 2% to 22% of household samples in rural Senegal (65), which correlated with the prevalence of Q fever cases in the population. Consumption of unpasteurized raw milk may be a source of human contamination, as *C. burnetii* has been detected in up to 63% of cattle milk samples in

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Nigeria (59). A few *C. burnetii* genotypes (including genotypes 2, 6, 16, 19, 30, 35, 36, and 52) have been characterized in Africa, mainly in ticks (56, 66). Only genotypes 19 and 35 have been detected so far in humans (56).

Q fever is not commonly considered a tropical disease. Clinicians should think of this diagnosis in febrile patients coming back from African countries. The fact that it has been reported in travelers returning from a safari tour illustrates this aspect (67).

Q fever outbreak in the Netherlands. Between 2007 and 2010, the Netherlands faced the largest Q fever outbreak ever reported, with more than 4,000 reported cases and an estimation of probably more than 40,000 total cases (4, 68, 69). The outbreak occurred in a population with low previous Q fever seroprevalence (2.4%) (70). The regions with the highest rates of infection were the Noord-Brabant province in the southern part of the country and the provinces of Gelderland and Limburg (71). Noord-Brabant province has 2.4 million inhabitants and 6.4 million farm animals, and goat farming is concentrated in this region. The outbreak correlated with the development of intense dairy goat farming in proximity to urban areas (72). The importation of a huge number of animals, with a 75-fold increase in the goat population between 1985 and 2009, could have contributed to the introduction of *C. burnetii*-infected animals in this country (4). Thanks to a retrospective study, it is now known that the epizooty had begun in 2005, with abortion rates above 60% on some farms (73). Thus, the combination of a large number of infected animals, the location of farms near populated areas, a lack of surveillance, and the low level of immune protection in this population probably explain the magnitude of the Q fever epidemic.

A public health strategy for controlling the outbreak had to be developed by Dutch authorities and was implemented in the spring of 2008. The notification of abortion cases in herds and vaccination became mandatory in June 2008 for goat or sheep farms with more than 50 animals in affected areas (74). Due to the inefficiency of these measures, and while fatal cases in humans were being reported, it was decided in December 2009 to systematically cull gestating goats and ewes, leading to the culling of more than 50,000 goats (75). In 2010, the number of reported cases in humans began to decrease, probably due to these veterinary measures and to increasing the immunization of the population.

As a consequence of the epidemic, many patients with underlying cardiac valve (or vascular) defects or prostheses could have been exposed to *C. burnetii* and might develop life-threatening endocarditis or vascular infections (76–78). Also, a high number of pregnant women could have been exposed to *C. burnetii* and might develop obstetrical complications and spontaneous abortions (79). Therefore, the long-term public health consequences of the Q fever epidemic, especially the potential development of a high number of persistent infections, are still a major concern.

This exceptional outbreak highlights that despite the microbiological knowledge about *C. burnetii* infection, its sudden emergence and spread cannot be predicted.

Hyperendemic Q fever in Cayenne, French Guiana. In Cayenne, the capital city of French Guiana, *C. burnetii* causes 24% of community-acquired pneumonia (CAP), which is the highest prevalence ever reported in the world (80). The first Q fever case was reported in 1955 in Cayenne, in a slaughterhouse worker (81). Sporadic cases were then reported in the following 4 decades. In the 1990s, a dramatic increase in the incidence of Q fever was observed, with seroprevalence rates rising from 2% in 1992 to 24% in 1996 in a cohort of febrile patients (3). The same year, a patient died of respiratory distress syndrome due to acute Q fever (82). The incidence continued to rise, with up to 150 cases per 100,000 inhabitants in 2005 (3). The majority of cases (81%) occurred in the Cayenne area and its suburbs, in contrast with the usual rural distribution of the disease. However, a more sensitive analysis of the incidence found a heterogenous distribution, with seven areas of high incidence. In these areas, rainforest hills are near houses (83). Living near the forest and seeing wild animals (bats or marsupials) around the house were risk factors for Q fever (82). No classical at-risk exposure was found in acute Q fever cases in Cayenne. Low Q fever seroprevalence was found in cattle, sheep, and goats, as well as in tested pets (82). In an initial study performed by Gardon et al.,

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no significant seroprevalence was observed among wild mammals and arthropods, so that no reservoir was identified (82). In a recent study, *C. burnetii* was detected by qPCR in the ticks, spleen, and stools of a dead three-toed sloth in Cayenne (84). In addition, the incidence of acute Q fever in Cayenne is correlated with the rainy season, and we observed a 1- to 2-month lag in the correlation between the incidence of acute Q fever and the number of births of three-toed sloths in Cayenne (85). Also, a retrospective study of an outbreak in a military camp in Cayenne in 2013 found that having carried a three-toed sloth in the arms in the month preceding symptoms was an independent risk factor for acute Q fever (unpublished data).

The same study found that 100% of cases (defined by a positive serology and/or qPCR in blood) in this military camp were symptomatic, which is an exceptional feature compared to the usual rate of 60% symptomatic cases found in most areas of endemicity. Clinically, the pneumonias caused by *C. burnetii* in Cayenne show a more severe initial presentation, with more frequent headaches, chills, and night sweats than pneumonia caused by other microorganisms (80). Moreover, patients tend to exhibit higher levels of phase I IgG, despite the acute presentation. These atypical features seem to be correlated with the presence of a single clone of *C. burnetii*, MST 17, which to date has only been isolated from this area. This clone also appears to be more virulent in animal models (see Pathophysiology section below).

The Reservoirs

Coxiella burnetii can infect a broad range of vertebrate and invertebrate hosts (86–88). Also, the bacterium can persist for prolonged periods in the environment, owing to a pseudosporulation process. Among mammals, cattle, sheep, and goats are the most frequent reservoir leading to human transmission. However, wildlife can also constitute a reservoir, as illustrated by an acute Q fever case reported after contact with kangaroos and wallabies in Australia (89) or by the involvement of the three-toed sloth in Cayenne (84). *C. burnetii* has also been isolated in many tick species, suggesting that these arthropods play a role in the transmission of the bacterium. Finally, it has been shown that *C. burnetii* is able to grow within amoebae, suggesting a participation of these hosts in the environmental persistence of the bacterium.

Domestic ruminants. Since domestic ruminants are the main reservoir responsible for human outbreaks, the detection and control of infected herds are important issues. However, the identification of infected animals is challenging, because dairy animals can shed the bacterium without being symptomatic. The main clinical manifestations in ruminants are reproductive disorders such as infertility, stillbirth, abortion, endometritis, and mastitis (87). Increased abortion rates in infected caprine herds have been described, with up to 90% abortions in pregnant animals (90). Infected females shed a huge amount of bacteria in birth products and in urine, feces, and milk. This shedding can persist for several months in vaginal mucus, feces, and milk (91). In nonpregnant animals, *C. burnetii* infection is most frequently asymptomatic.

Rodolakis et al. have compared the shedding of *C. burnetii* in bovine, caprine, and ovine herds from France (44). Cows were mostly asymptomatic and shed *C. burnetii* mainly in milk, with a few of them (5%) shedding in vaginal mucus. Ovine flocks were the most heavily infected, with shedding of the bacterium in feces, vaginal mucus, and milk, and ewes shed the bacterium for up to 2 months of follow-up. Goats shed the bacterium mainly in milk, and a minority shed *C. burnetii* in vaginal mucus or feces (44). These differences in shedding routes could explain why human outbreaks are most frequently related to small ruminants rather than to bovine herds, which is what was observed in the Netherlands. Interestingly, in the same study, seropositivity for *C. burnetii* was not strongly correlated with shedding of the bacterium. Fewer than 10% of ovine flocks shedding *C. burnetii* tested positive for *C. burnetii* antibodies by enzyme-linked immunosorbent assay (ELISA), and the proportions were 35% and 50% for bovine and caprine herds, respectively (44). As a consequence, serology does not seem to be an efficient tool in estimating the real rate of contamination of herds. For

monitoring purposes, PCR testing of bulk tank milk is a useful method to detect shedding of *C. burnetii* at the farm level (92).

Preventive veterinary measures are a key point in the control of Q fever. Antibiotic treatment with oxytetracycline (20 mg/kg) during the last months of pregnancy has been proposed to reduce abortion rates and *C. burnetii* shedding in pregnant animals (93). However, this strategy is not always effective, since Astobiza et al. found no difference in rates of bacterial shedding between treated and untreated sheep (94).

Vaccination is another available option to decrease abortion rates and spread of the infection. The phase I vaccine Coxevac has been effective in decreasing abortion rates and bacterial load in vaginal mucus, feces, and milk in goats (95). The vaccination should be administered in noninfected ruminants before their first pregnancy (74). When ruminants are already infected, vaccination cannot prevent abortion (90, 96). Also, standard hygiene measures should be taken to prevent dissemination of *C. burnetii* in the environment and between animals. Infected parturient females should be isolated from the herd, and placentas and fetuses should be collected and destroyed by burning or burying (90). Moreover, the spreading of manure in infected farms should be avoided during windy conditions. Animals exhibited at fairs should also be carefully selected. In Germany, an infected ewe lambing in a farmer's market led to an outbreak of 299 Q fever cases in the people present (97). In Briançon, a town in France located in the Alps, the investigation of a slaughterhouse-related outbreak found that contamination resulted from airborne transmission of contaminated sheep waste, facilitated by the wind caused by a nearby heliport (98, 99). Finally, when no preventive measures can be applied and if too many contaminated animals are involved, culling of herds is the ultimate solution, and this was performed in the Netherlands to control the epidemic (90).

The main routes of introduction of *C. burnetii* on a farm are the aerosolized spore-like forms transported by the wind (100) and the introduction of an infected animal. Then, depending on the size and immune status of the herd, the spread of the bacterium can lead to "abortion storms," such as what occurred in the Netherlands in 2005 and 2009 (90).

Role of ticks. *C. burnetii* strains were first isolated from hard ticks: *Dermacentor andersoni* (Nine Mile isolate) collected in Montana (101) and *Haemaphysalis humerosa* from Australia (102). Hematophagy is the essential factor for the acquisition of *C. burnetii* by arthropods. Following the "classical" epidemiological pattern of zoonotic infections, *C. burnetii* likely circulates among animals with the help of hematophagous arthropod vectors. However, in contrast to the case for most vector-borne diseases, the presence of an arthropod vector is not necessary for the transmission of the infectious agent from the reservoir to host mammals.

Another important aspect of the epizootic cycle of *C. burnetii* is the absence of any vector specificity. This bacterium was isolated from more than 40 hard tick species, at least 14 soft tick species, and many other arthropods, including bed bugs, flies, and mites (Table 1). Even more arthropod species (including human lice and fleas) were shown to be susceptible to *C. burnetii* infection under experimental conditions (103), although they were not able to transmit the agent to experimental animals or to their progeny.

(i) Susceptibility of arthropods. Hematophagous arthropods at all stages of their development can be easily infected with *C. burnetii* when taking a blood meal from an infected mammal (104). However, in experimental situations, not all ticks feeding on a *C. burnetii*-infected animal become infected; e.g., in the case of a *Haemaphysalis humerosa* infection model, only half of the ticks became infected (102, 105). A similar situation was described for the experimental infection of *Ixodes ricinus* and *Ornithodoros papillipes* ticks fed on infected guinea pigs (104).

(ii) Localization inside the tick. The first experiments on the localization and dissemination of *C. burnetii* in ticks were performed by D. J. W. Smith on *Haemaphysalis humerosa* and *Haemaphysalis bispinosa* (105, 106). Bacteria were abundant in the epithelial cells and lumen of the gut. However, the transovarial transmission

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TABLE 1 Arthropods from which *C. burnetii* has been isolated

Arthropod species from which strain was isolated	Host(s)	Reference(s)
Hard ticks (Ixodidae)		
Amblyomma americanum	Deer, cattle	103
Amblyomma cajennense	Dogs	670
Amblyomma flavomaculatum	Monitors	103, 671
Amblyomma nuttalli	Reptiles	103, 671
Amblyomma paulopunctatum	Suidae	103, 671
Amblyomma splendidum	Ungulates	103, 671
Amblyomma triguttatum	Kangaroos	672
Amblyomma variegatum	Cattle	103, 109, 671
Dermacentor andersoni	Deer, cattle	673
Dermacentor marginatus	Deer, cattle	(186)
Dermacentor nuttalli	Ruminants	(186)
Dermacentor occidentalis	Deer, cattle	103
Dermacentor silvarum	Deer, cattle	(186)
Haemaphysalis bispinosa	Kangaroos	674
Haemaphysalis humerosa	Marsupial bandicoot	102
Haemaphysalis leachi	Dogs	103, 671
Haemaphysalis leporis-palustris	Hares	103
Haemaphysalis punctata	Rodents	103, 186
Hyalomma asiaticum	Ruminants	104, 675
Hyalomma anatolicum	Cattle	104
Hyalomma marginatum	Cattle	103, 671
Hyalomma detritum	Ruminants	103, 104
Hyalomma dromedarii	Camels	103, 104
Hyalomma excavatum	Cattle	103
Hyalomma lusitanicum	Horses	103
Hyalomma plumbeum	Cattle	103, 108
Hyalomma scupense	Cattle	104
Ixodes crenulatus	Weasels	104
Ixodes dentatus	Rodents, rabbits	103
Ixodes frontalis	Birds	104
Ixodes holocyclus	Dogs, rodents	674
Ixodes lividus	Birds	104
Ixodes persulcatus	Ruminants	104, 676
Ixodes redikorzevi	Argali	677
Ixodes ricinus	Ruminants	23, 103
Ixodes trianguliceps	Rodents	104
Rhipicephalus annulatus	Cattle	104
Rhipicephalus bursa	Cattle	103, 108
Rhipicephalus cuspidatus	Aardvarks	103, 671
Rhipicephalus decoloratus	Cattle	103, 671
Rhipicephalus sanguineus	Dogs	671, 678
Rhipicephalus simus	Carnivores	103, 671
Rhipicephalus turanicus	Ruminants	104
Soft ticks (Argasidae)		
Argas persicus	Birds	104, 671
Argas reflexus	Birds	104
Argas vespertilionis	Bats	679
Ornithodoros alactagalis	Rodents	104
Ornithodoros erraticus	Rodents	103
Ornithodoros gurneyi	Kangaroos	105
Ornithodoros hermsi		680
Ornithodoros lahorensis		103
Ornithodoros moubata		103, 113, 680
Ornithodoros papillipes		679
Ornithodoros sonrai	Rodents, insectivores	56
Ornithodoros tartakovskyi	Rodents	679
Ornithodoros turicata		110
Otobius megnini	Horses, cattle	103
Other arthropods		
Musca domestica		103, 186
Cimex lectularius	Humans	186, 681
Hematophagous *Mesostigmata* mites: at least 14 species, including *Liponyssoides sanguineus*, *Ornithonyssus bacoti*, *Haemolaelaps glasgowi*, *Dermanyssus hirundinis*, and *Haemogamasus nidi*	Rodents	104, 186, 682

of *C. burnetii* was reported for *H. humerosa*, suggesting that these ticks could serve as a long-time reservoir for this pathogen. Using an *Ornithodoros moubata* model, von Weyer later showed the generalized dissemination of *C. burnetii* in this tick's body, affecting the gut, hemocytes, salivary glands, and ovaries (107). Tarasevich, working on *Haemaphysalis plumbeum* ticks infected intracoelomically with *C. burnetii*, demonstrated that this bacterium was visible in hemocytes for several hours postinfection and was detectable in salivary glands and ovaries from the third day postinfection (108). Similar data were observed in different experiments on *Dermacentor nuttalli*, *Haemaphysalis asiaticum*, and *Ornithodoros papillipes* (103, 104).

(iii) Survival of *C. burnetii* in ticks. It is clear that *C. burnetii*, once infecting tick cells, is capable of remaining viable inside the tick's body for a very long time, between 200 (109) and 1,000 days, depending on the tick species (110). However, in several cases, much longer survival times have been reported: 1,301 days in *O. moubata* (107) and even 6 to 10 years in *O. papillipes* (104). Low temperatures, starvation, and feeding on an immunized host do not influence the viability of *C. burnetii* (104).

(iv) Transstadial and transovarial transmission. Most soft and hard ticks transmit *C. burnetii* transstadially in 100% of cases (104). Similarly, it is thought that most hard and soft ticks are able to transmit *C. burnetii* transovarially. Several exceptions include *Ixodes holocyclus*, *Ornithodoros hermsi*, and *Ornithodoros turicata* (104). However, transovarial transmission is not very effective and may vary from 30 to 60% (102, 104, 107).

(v) Excretion of *C. burnetii*. Massive excretion of highly infective phase I *C. burnetii* (11) in tick feces occurs on the skin of the animal host at feeding time. This is the direct consequence of the multiplication of *C. burnetii* primarily inside gut epithelial cells. Feces may contain a huge number of bacteria, from 10^3 to 10^8 in different experiments (102, 104, 106, 111). Excreted bacteria stay viable in tick feces for many days (up to 635), although infectivity decreases with time (104). As for other bacteria excreted in arthropod feces, abraded skin may serve as a portal of entry for infection (112).

Soft ticks may also excrete *C. burnetii* in coxal fluid (6, 20). This may even be the most important method of *C. burnetii* excretion for soft ticks with no direct connection between the rectum and the midgut, such as in *O. moubata* (113).

(vi) *C. burnetii* influence on tick fitness. No deleterious effects of *C. burnetii* in ticks has been reported (104). Slight metabolic changes between infected and noninfected ticks, such as oxygen consumption and CO_2 excretion, have been noted. *C. burnetii* infection is considered to be harmless to ticks (104).

In livestock, ticks are probably nonessential for transmission of *C. burnetii* (103). However, they seem to play a role in the transmission of *C. burnetii* among other vertebrates (rodents, wild birds, and lagomorphs) (103, 114).

Detection and strain isolation of *C. burnetii* from bed bugs, lice, and flies (Table 1) (103) have been reported, but the role of these arthropods in the natural cycle of *C. burnetii* is unknown.

Role of free-living amoebae. *C. burnetii* is an intracellular bacterium that multiplies in monocytes and macrophages of infected hosts (115). Prokaryotes colonized Earth before eukaryotes, and unicellular eukaryotes emerged before multicellular ones. It has been emphasized that most intracellular bacteria infecting humans and animals are able to multiply within unicellular protozoa such as amoebae (116). Because these protozoa are widespread in nature, many bacteria have developed mechanisms to resist the phagosomal pathway of amoebae. These microorganisms have then used similar mechanisms to resist attack by the phagocytic cells of multicellular eukaryotes. *Legionella pneumophila*, the agent of legionellosis, is a paradigm for such an adaptive evolution (117). This bacterium can multiply in the vegetative form of amoebae. Under deleterious environmental conditions, *L. pneumophila* survives in encysted amoebae until they recover their vegetative form. Thus, amoebae allow long-term persistence of *L. pneumophila* in water and soil environments. *In vitro*, *C. burnetii* can multiply in amoebae, which may represent a long-term environmental reservoir for this bacterium (118). A report from Amitai et al. (119, 120) of a Q fever outbreak in an Israeli school occurring after exposure to an air conditioning system favored such a hypothesis.

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However, further studies are needed to isolate *C. burnetii*-infected amoebae from the natural environment.

Routes of Human Infection

A single study of experimental infection with *C. burnetii* in human volunteers was performed in 1950 in Portugal (121). This very old work is interesting because it summarizes the main possible routes of human infection. Fifty-one human volunteers took part in the experiment. Ten volunteers had intranasal inoculation, 11 ate infected food, and 29 were infected intradermally. The most efficient route of infection was intradermal inoculation, which resulted in 100% seroconversion. Three patients (27%) seroconverted after consumption of infected food and two patients (20%) after intranasal inoculation.

Aerosols. Most human infections occur after inhalation of infected aerosols of *C. burnetii* (86–88). Infection may occur after direct exposure to infected animals and their products (placenta, abortion products, hides, wool, manure, etc.), especially at the time of parturition or slaughtering (49–51). Because *C. burnetii* may persist for prolonged periods in the soil, these aerosols may also be produced long after the release of bacteria by infected animals. Moreover, bacterial aerosols can be delivered for at least 30 km by the wind (122), resulting in Q fever cases far away from the primary contaminated areas. Thus, Q fever cases are often diagnosed in persons with no recent contact with animals.

Digestive route. The hypothesis that consumption of dairy products from *C. burnetii*-infected animals can lead to foodborne Q fever in humans is controversial (91). In an early study, Huebner and Jellison found that pasteurization of dairy products could eliminate *C. burnetii* from infected milk (123). Other studies have shown higher seroprevalence rates and clinical disease in patients consuming raw milk (124). However, a significant bias of these studies is that people consuming raw milk may live more frequently in rural areas and be in contact with ruminants, so that contamination could also be the result of aerosol inhalation. However, during an epidemic in Newfoundland, Canada, eating pasteurized goat cheese was an independent risk factor for acute Q fever (125). Also, in an old study performed in a prison, *C. burnetii* serology was found to be more frequently positive in persons consuming raw milk than in persons who did not (126). Conversely, no seroconversion or clinical illness was observed in another study involving 34 volunteers consuming raw milk (127). *C. burnetii* DNA has been detected in up to 64% of dairy products in France (124) and more frequently in cow dairy products. However, no viable bacteria could be isolated from cheese and yogurt in that study. Consequently, even if the digestive route of contamination does not constitute a major public health threat, it may play a significant role in *C. burnetii* transmission.

Ticks. Although this mode of contamination has not been proven in humans, ticks may play a role in the transmission of *C. burnetii* infection. This is illustrated by the detection of *C. burnetii* coinfection with other arthropod-borne pathogens in ticks. In Italy, 85 *Rhipicephalus turanicus* and 33 *Ixodes ricinus* ticks were collected from a public park in Rome (128). Coinfection with *Rickettsia* of the spotted fever group and *C. burnetii* was observed in 5 (5%) *R. turanicus* ticks. Double infection with *C. burnetii* and *Borrelia burgdorferi* was detected in 7 (21%) of *I. ricinus* ticks, and a positive statistically significant correlation between these two pathogens was observed. These findings of different microorganisms in ticks from urban areas suggest a common mode of transmission for both human pathogens via arthropods (128).

Human-to-human transmission. Q fever pneumonia is considered a noncommunicable disease, although a recent case of respiratory nosocomial spread was reported (129). Anecdotal cases of human-to-human transmission through infected aerosols have been reported after autopsies (88). Birth products from infected parturient women are also a source of infection in obstetrical wards. A case of *C. burnetii* pneumonia was diagnosed in an obstetrician 7 days after he delivered the infant of an infected woman (130). Also, nosocomial transmission between two pregnant women sharing the same

room has been reported (131). In this case, the most probable route of infection was vaginally excreted aerosolized infectious particles.

C. burnetii infection through transfusion of blood collected from Q fever patients with bacteremia is plausible, since the bacterium can survive in stored human blood samples (132). In the Netherlands, screening of blood donors after the outbreak found 4.4% of donors with positive phase II antibodies (133). Another study found that during the outbreak, the probability of a donor being infected was estimated at 260/100,000 donors (134). As a consequence, systematic donor screening was the most useful strategy for reducing the risk of transmission through blood transfusion. A single case of Q fever was also reported after bone marrow transplantation (135). Another anecdotal case of possible sexual transmission of *C. burnetii* from a farmer to his wife has been reported (136).

Seasonality Patterns and the Role of Wind

In areas of endemicity, the Q fever incidence is variable through the year. Seasonal patterns have been observed. In the southeast of France, Tissot-Dupont et al. found a correlation between the incidence of the disease, sheep densities, and the local mistral wind in the city of Martigues (46). During the winter in 1998 to 1999, the high incidence of acute Q fever in this region was associated with an increased frequency of mistral wind shortly after the lambing season, 1 month before disease onset (122). In the UK, unusual wind speed from the south was observed shortly before a large epidemic in Birmingham (137). In Germany, a long-term survey on Q fever has revealed a changing pattern of seasonality, from winter-spring to spring-summer (138). This evolution is probably due to changes in sheep husbandry. Winter-spring lambing is associated with a type of nomadic husbandry that has considerably decreased throughout the years in this country. In Croatia, acute Q fever cases have been observed mainly in the spring.

In tropical areas, two examples suggest that acute Q fever incidence may be related to the rainy season. In Queensland, Australia, a clear seasonal peak of acute Q fever cases was observed in May, 3 months after a peak February rainfall (139). In Australia, the rainy season corresponds to an increase in the populations of macropods (wallabies and kangaroos) and other wildlife that potentially plays a role in the spread of the bacterium. Rates of up to 20.8% seroprevalence for *C. burnetii* have been observed in macropods (140). In Cayenne, acute Q fever incidence is the highest in July, with a 2-month correlation following a peak in rainfall in May, which is the breeding season of the three-toed sloth, which is a probable wild reservoir of the infection (85).

GENOMIC ASPECTS

The first genome of *C. burnetii* was sequenced in 2003 (9). This event led to significant progress in many fields of study of this bacterium. In particular, the intracellular nature of *C. burnetii* made the search for virulence determinants very difficult. Genomics, and more particularly comparative genomics studies, have demonstrated that the word "Q fever" covers a large range of epidemiological and pathogenicity characteristics, depending mainly upon the genetic characteristics of the *C. burnetii* strain involved.

Comparative Genomics and Pangenomic Analysis

The RSA493 strain, isolated from a tick in 1935, was the first strain to be sequenced (9). It revealed a QpH1 plasmid (37,393 bp) and a 1,995,275-bp chromosome. When comparing this genome with genomes from close relatives, including *Legionella*, *Chlamydia*, and *Rickettsia* species, several particularities were found. There were 719 hypothetical coding sequences, with no equivalent in gammaproteobacteria. There were more coding sequences than in the genomes of *Rickettsia prowazekii* and *Mycobacterium leprae*. The *C. burnetii* genome exhibited 83 pseudogenes, showing that genome reduction was in progress. It was also characterized by the presence of 29 insertion sequence (IS) elements, in favor of high genomic plasticity.

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Regarding metabolic pathways, the *C. burnetii* genome contained a high number of transporters, reflecting the intracellular lifestyle of the bacterium, which finds its organic nutrients within the eukaryotic host cell. In comparison with other intracellular bacteria, the central carbon metabolism and bioenergetics pathways were mainly intact. Also, many ionic exchangers were present, playing a role in the survival of the bacterium by detoxifying the phagolysosome-like vacuole where *C. burnetii* multiplies. Many genes were very similar to eukaryotic genes, with no equivalent in prokaryotes, similar to what has been found in *Legionella*. Also, a new family of ankyrin repeat-containing proteins (Anks) was reported. Anks are mediators in protein-protein interactions and play a role in intracellular processes (141).

Since 2003, six new whole genomes of *C. burnetii* have become available in GenBank (142–144). Sixteen incomplete genomes can also be found (27, 47, 145–150). In 2009, Beare et al. performed a comparative genomic analysis of four strains and found a total of 125 pseudogenes, of which 65 were highly conserved, and 8 IS families (142). These features were consistent with genome reduction due to recent adaptation of *C. burnetii* to an intracellular lifestyle. More recently, D'Amato et al. performed a pangenomic analysis of the seven sequenced strains of *C. burnetii* (144). The Dugway strain exhibited the largest genome (2,158,758-bp chromosome and 54,179-bp QpDG plasmid). Cb175 from Cayenne, French Guiana, had the smallest genome, due to a unique 6,105-bp deletion in the coding region for the type 1 secretion system (T1SS) (1,989,565 bp chromosome, 37,398 bp QpH1 plasmid). Some strains had one of the plasmids QpRS, QpH1, and QpDV. The others had plasmid sequences integrated into the chromosome. A BLAST score ratio (BSR) analysis allowed the description of core accessory and unique genes. The core genome/pangenome ratio was 96%. A total of 13,542 core genes (shared by all strains), 498 accessory genes (found in some strains), and 88 unique genes (found in a single strain) were found (144). Seventy-four unique genes were found in the Dugway strain, 13 in the Q212 strain, and a single gene in the RSA331 strain.

The conclusion from this work is that *C. burnetii* strains share strong genomic similarity, with a closed pangenome. However, some particularities were observed regarding two epidemic strains, reflecting the links between genomic characteristics and virulence.

Genome and virulence. (i) Cb 175, a strain from Cayenne, French Guiana. The genome sequencing of strain Cb 175, isolated from a patient with endocarditis living in Cayenne, French Guiana, revealed an unexpected feature: a deletion of 6,105 bp resulting in a large genome reduction compared to the Nine Mile strain (139). A specific qPCR system was established with primers and probes targeting DNA sequences adjacent to the deleted region. This qPCR test was positive for all other clinical strains isolated from French Guiana. Conversely, this deletion was not found in any of the 298 *C. burnetii* isolates from other parts of the world (8/8 versus 0/298; $P < 0.001$). Alignment of the missing region with the genome of *Legionella pneumophila* revealed the presence of a conserved region of a type 1 secretion system (T1SS). Interestingly, the genes *lssB* and *lssD*, which were absent in Cb175, were also missing in *Legionella longbeachae* (144).

Several conclusions can be drawn from this study. First, genome reduction has been observed in many highly epidemic strains (151). For species like *Rickettsia* or *Mycobacterium* spp., hyperpathogenicity is driven by deletion of nonvirulence genes rather than by acquisition of virulence factors (152, 153). Cb175 and other strains of the genotype MST 17 specific for French Guiana are the most virulent strains ever described. They cause the highest prevalence of community-acquired pneumonia in the world. Consequently, the observed genome reduction is probably a mechanism leading to increased virulence in this *C. burnetii* clone. Also, it has been shown that highly pathogenic species have fewer secretion system proteins than their nonepidemic relatives (154). The fact that the deletion in Cb175 is located in the region of genes involved in the T1SS is consistent with this phenomenon. The role of T1SS in *C. burnetii* is not known, but it plays a role in the internalization of *Legionella pneumophila* in its host cell (155).

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The exact pathophysiological consequences of the genetic deletion observed in both *L. longbeachae* and *C. burnetii* are currently unknown, and further experimental studies are warranted.

(ii) Strain Z3055, a strain close to the strain of the Netherlands outbreak. To date, the complete genome sequence of NL-Limburg, the strain causing the Netherlands outbreak is not available (150). Therefore, we sequenced the Z3055 strain isolated from a German ewe, which belonged to the same genotype (MST 33) as NL-Limburg (150). Compared with other *C. burnetii* genomes available in GenBank, we found a high proportion of mutations in genes coding for ankyrin repeat domain proteins, membrane proteins, and proteins involved in translation and transcription (143). Thus, we hypothesized that these mutations may have changed surface antigens, as in the case of influenza virus. These modifications in surface antigens could have led to an absence of immune recognition in a naive population, allowing the rapid spread of this specific clone in the Netherlands.

(iii) Strain DOG UTAD, from Canada. The majority of strains isolated so far in Canada belong to the MST 21 genotype. An interesting feature of Q fever outbreaks in Canada is the frequent exposure of patients to parturient pets (cats or dogs) (48, 156, 157). In 1994, three members of the same family had acute Q fever after assisting with the parturition of their dog in Nova Scotia (48). The whole genome of the strain isolated from this outbreak was sequenced. It was very close to the Q212 strain, which had been isolated from a patient with *C. burnetii* endocarditis in Canada. DOG UTAD was plasmidless and showed 70 mutations, of which 47 were nonsynonymous compared to Q212. Both strains belong to the same genotype, MST 21, which has been isolated only in Canada to date (47).

(iv) Plasmids and virulence. A correlation between plasmid types and virulence was proposed previously. In this hypothesis, strains with a QpH1 plasmid were associated with "acute Q fever" and strains harboring QpRS were associated with "chronic Q fever" (158). The substratum for such a hypothesis was that specific sequences in plasmids correlated with pathogenicity. For example, the gene *cbbE′* was found to be specific for QpRS, and *cbhE′* was found to be specific for QpH1 (159, 160). However, this hypothesis was ruled out by several studies. First, Stein and Raoult showed that *cbhE′* was not systematically detected in strains from "acute Q fever" and conversely could be detected in isolates from chronic Q fever (161). The description of a new plasmid type, named QpDV, which can be associated with both forms of the disease, has been another argument against the existence of plasmid pathotypes (162). Finally, Thiele and Willems also found endocarditis isolates harboring QpH1 sequences (163). Nevertheless, in a recent study, Angelakis et al. found that the QpDV plasmid was associated with strains causing abortion (164). However, this correlation could be related to the confounding factor represented by genotype, so further studies are needed to confirm this point.

***C. burnetii* genotyping.** Genotyping of bacteria is a key tool in the understanding of the epidemiology of infectious diseases. With regard to a zoonosis like Q fever, it is of tremendous importance, helping to find the animal source of human outbreaks. In initial studies, 16S rRNA sequencing, 16S-23S rRNA sequencing, RNA polymerase β-subunit (*rpoB*) sequencing, and internal transcribed spacer (ITS) sequencing were used as epidemiological markers but showed insufficient discriminant power for *C. burnetii* genotyping (165). Then, restriction fragment length polymorphism (RFLP) analysis and PCR-RFLP targeting several genes were developed, but these methods could not be used on a routine basis (165). Comparative genomic hybridization (CGH), thanks to whole-genome microarray techniques, was performed when genome sequences became available. Different "genomotypes" were defined based on polymorphisms in plasmid open reading frames (ORFs) and chromosomal sequences (166), but this method is time-consuming. Loftis et al. have tried to describe the diversity of strains by detection of different plasmid sequences, but this has a poor discriminant power (167). Currently, the three main discriminant genotyping methods used are multiple-

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locus variable-number tandem repeat (VNTR) analysis (MLVA), multispacer sequence typing (MST), and single nucleotide polymorphism (SNP) genotyping.

(i) VNTR-MLVA genotyping. VNTR-MLVA genotyping was established by Svraka et al., who amplified VNTR sequences from 21 *C. burnetii* isolates (168). They identified five main clusters and nine MLVA types. Arricau-Bouvery et al. then analyzed 42 isolates and found 36 MLVA types. They proposed using two panels of markers to have a better AQ: G discriminatory power (169). In the literature, there are 17 references for studies that have used this genotyping method, almost all from Europe and mainly among ruminants (66, 73, 170–184). The clone causing the Netherlands outbreak was identified as CbNL01 using this method, and a very similar clone was detected in goats in Belgium (178). However, MLVA is based on the analysis of relatively unstable repetitive DNA elements and can produce results that are too discriminatory (185). Moreover, it significantly lacks interlaboratory reproducibility (185).

(ii) MST genotyping and "geotyping." MST genotyping was introduced by Glazunova et al., who identified 10 highly variable spacers located between ORFs (186). This typing method identified 30 different genotypes and three monophyletic groups among 173 *C. burnetii* isolates. These groups were partially correlated with plasmid types. The first group contained strains with the QpDV or QpRS plasmid, the second contained only strains with the QpH1 plasmid, and the third group contained plasmidless strains or strains with QpH1. This method is very discriminant and has been used most frequently in AQ: H different studies around the world. Seventeen studies have used MST genotyping from human, animal, or environmental *C. burnetii* strains (2, 56, 66, 84, 164, 172, 173, 176, 179, 183, 184, 186–191), providing a worldwide database allowing interlaboratory comparF1 ison (Fig. 1). MST genotyping helps to trace the spread of *C. burnetii* from one region to another and from animal reservoirs to humans. Some MSTs are present across the five continents, whereas others are very specific to epidemic situations. For example, MST 20 has been described in ruminants in Europe and in humans and ruminants in the United States, suggesting a spread of the disease by infected animals historically brought to the New World. In contrast, MST 17 has been isolated only from French Guiana to date, causing severe forms of the disease (192). In the Netherlands, MST genotyping identified MST 33 in goats and humans, allowing confirmation that the source of the epidemic was goat herds rather than cow herds, which predominantly harbored MST20 (176). The phylogenetic analysis showed that MST 33 may have spread from Germany to the Netherlands via France.

For these reasons, MST genotyping has been qualified as a "geotyping method" (Fig. 1). This "geotyping" scheme is still incomplete and has to be implemented in further studies to provide a comprehensive cartography of the genetic diversity of *C. burnetii*.

(iii) SNP genotyping. SNP genotyping was developed by a Dutch team during the outbreak in the Netherlands to provide a method directly applicable to animal and human samples without the need for enrichment by a culture step (193). Ten discriminatory SNPs were selected using five *C. burnetii* whole-genome sequences available in GenBank. (RSA493, RSA331, CbuG_Q212, Cbuk_Q154, and Dugway). Detection of SNPs by reverse transcription-PCR (RT-PCR) was performed in 14 human and 26 animal samples, allowing identification of five different genotypes. This method has also been used in Belgium and in the United States for livestock and tick strains (178, 194–196). Karlsson et al. developed an SNP genotyping method targeting 10 phylogenetically stable synonymous canonical SNPs (canSNPs) (197). Finally, Hornstra et al. developed an SNP method derived from MST genotyping (189). They extracted SNP signatures in MST loci and designed 14 SNP-base assays. These assays allowed genotyping of 43 previously untyped isolates when using classic MST, increasing the database available for worldwide comparison of "geotypes" (Fig. 1).

(iv) Other methods. Some other genotyping methods have been reported on the basis of small samples, giving very local information. In Spain, Jado et al. developed a genotyping method based on the detection of seven ORFs and on the presence or not of the acute disease antigen A (adaA) (198). They identified seven genomic groups and 10 different genotypes among 90 samples from ruminants and humans. In that study,

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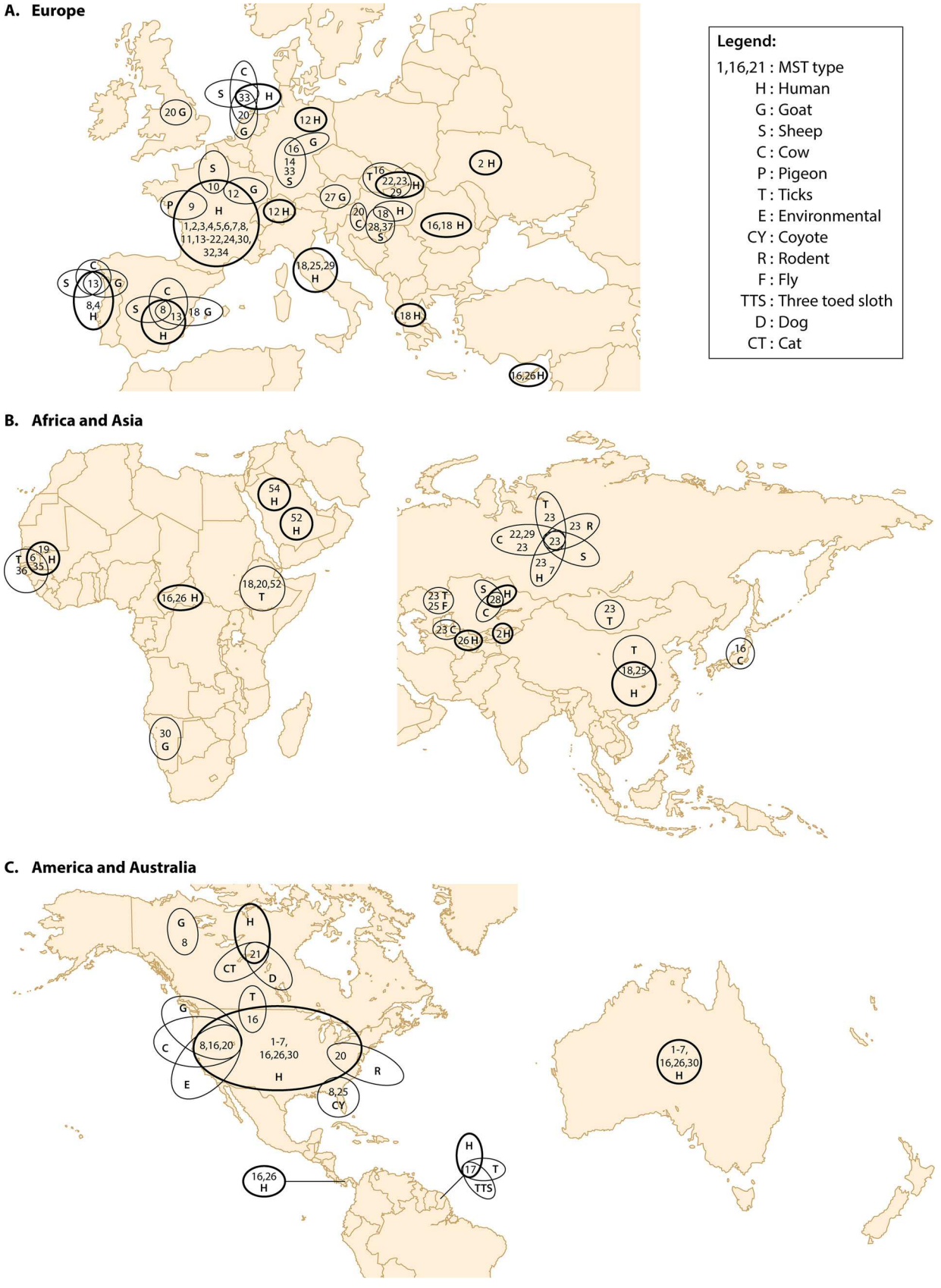

FIG 1 *C. burnetii* geotyping.

COLOR

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sheep, wild boars, goats, ticks, and rats shared common genotypes with humans, while cattle harbored a different one (198). In France, Sidi-Boumedine et al. used the randomly amplified polymorphic DNA (RAPD) method for genotyping of 10 isolates from goats, sheep, and cows (199). This method was more discriminant than the MLVA method among these strains.

Phylogenetic Aspects

Historically, *C. burnetii* was considered a member of the *Rickettsiaceae* family in the class of *Alphaproteobacteria*. Actually, the sequencing of the 16S rRNA gene has shown that it is a member of the *Gammaproteobacteria*, close to *Rickettsiella grylli* and *Legionella pneumophila* (200) (Fig. 2). For years, *C. burnetii* was the only species of the *Coxiella* genus, so that phylogenetic studies were not accurate. However, comparison with some close genera revealed interesting features. Like *Legionella*, *C. burnetii* can survive in amoebae (118). *C. burnetii* has a sterol-delta-reductase gene very similar to that of *Legionella drancourtii*. This gene is eukaryotic and is not present in other close relatives (118, 201). This suggests horizontal gene transfer between these two bacteria within the amoeba (201). Similarly, nontuberculous mycobacteria that can live in amoebae have a pyridine nucleotide disulfide oxidoreductase (pyr-redox) very close to that of *C. burnetii* (202).

Coxiella cheraxi, another member of the genus, was described in 2000, showing 95.5% similarity with *C. burnetii* in 16S rRNA (203). To date, its genome sequence is still not available. The most recent advances in the description of *C. burnetii* phylogeny have resulted from the study of *Coxiella*-like endosymbionts from ticks (204–207). These microorganisms have been identified mainly thanks to 16S rRNA sequencing. A *Coxiella*-like endosymbiont from the tick *Amblyomma americanum* (CLEAA) has been studied by Smith et al. (204). CLEAA is phylogenetically closely related to *C. burnetii* but has a reduced genome and does not seem to derive from it. These two species are both derived from a probable common ancestor (204). Recently, the phylogenetic analysis of a third species, the *Coxiella*-like symbiont of *Rhipicephalus turanicus* ticks (CRt), suggested that it also shares the same common ancestor (207). However, compared to these two other species, the genome of *C. burnetii* shows a high number of unique genes, suggesting gene gain events in its lineage (207). A deeper analysis among *Coxiella*-like tick endosymbionts has been performed by Duron et al. (205). They combined a new multilocus typing method and whole-genome sequencing for the phylogenetic analysis of *Coxiella*-like DNA from 637 specimens of ticks. They found a high diversity of *Coxiella*-like organisms, which could be divided in four clades (A to D) (Fig. 3). These *Coxiella*-like microorganisms were widely distributed among the different tick species, suggesting a long coevolution of *Coxiella* and ticks. This work also revealed that *C. burnetii* belonged to a unique subclade within clade A of *Coxiella*-like endosymbionts associated with soft ticks. Thus, *C. burnetii* has probably recently emerged from a tick-borne ancestor.

The genomes of *Coxiella*-like organisms are small (0.7 and 1.7 Mbp) (207). All known genomes of C. *burnetii* are larger (2.0 to 2.1 Mbp) than genomes of *Coxiella*-like organisms. Thus, it has been proposed that *C. burnetii* recently evolved to vertebrate pathogenicity from an inherited endosymbiont of ticks, due to spontaneous mutations or horizontal gene transfers from pathogens that coinfected the same tick or vertebrate host (205, 206). We recently detected a new *Coxiella*-like species, "*Candidatus* Coxiella massiliensis," from ticks and skin biopsy specimens of patients presenting with eschars and SENLAT (scalp eschar and neck lymphadenopathy after tick bite). This microorganism may be one of the etiological agents of this syndrome. Further studies are needed to know if "*Candidatus* Coxiella massiliensis" is a representative agent of the evolution from tick endosymbiont to human pathogen (208).

Genetic Transformation

The first genetic transformation experiment with *C. burnetii* was achieved in 1996 by Suhan et al. (209). Before that, the same authors performed cloning in *Escherichia coli*

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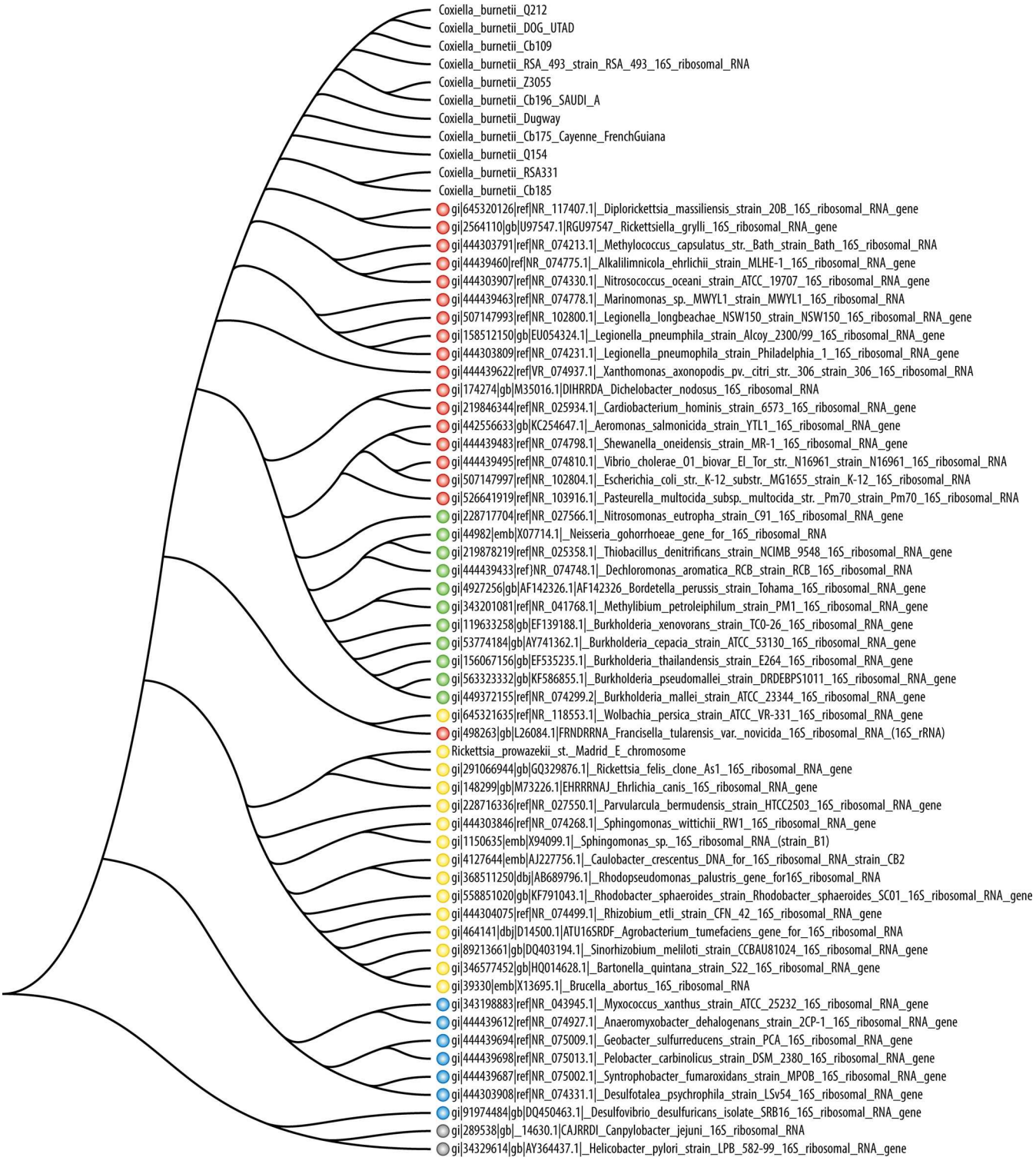

FIG 2 Phylogeny estimation of proteobacterial 16S rRNA genes. (Adapted from reference 165 with permission of Future Medicine Ltd.)

to identify an autonomous replicating sequence (ARS) of 403 bp (210). This sequence was used to transform *C. burnetii* to ampicillin resistance. The transformation was successfully achieved by using a plasmid containing the ARS and a ColE1-type replicon encoding beta-lactamase, which was introduced with electroporation (209). Some years later, Lukacova et al. proposed improving the technique by adding a green fluorescent protein as a marker of transformation (211). Then, in 2009, Beare et al. reported the first

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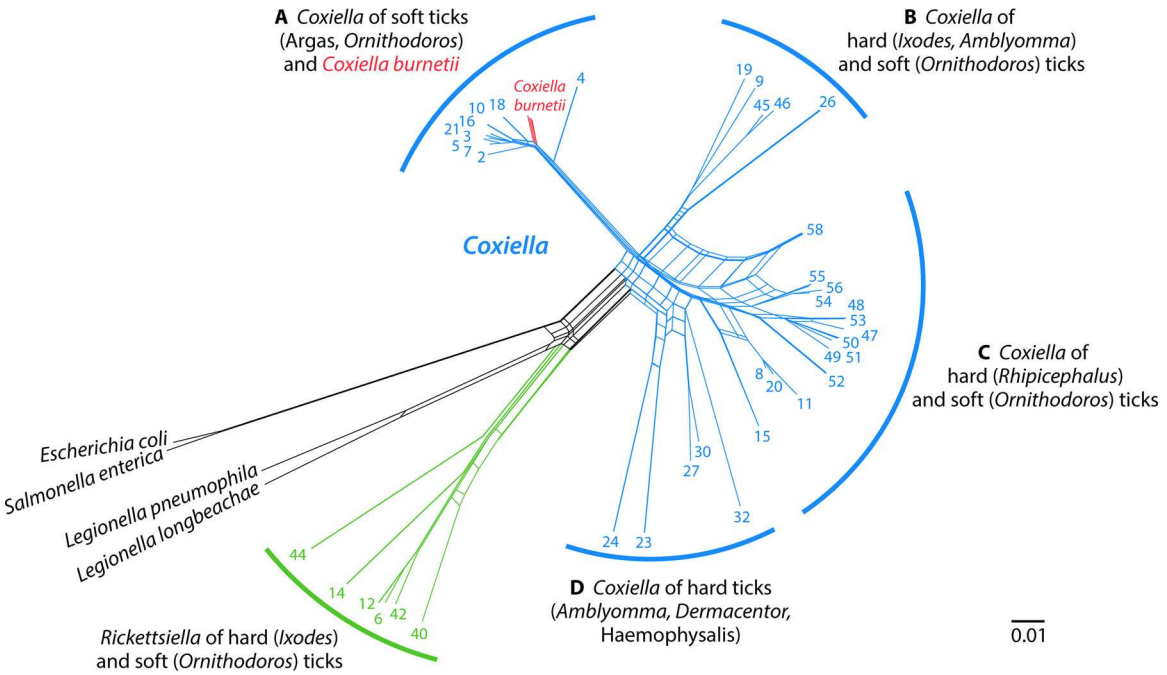

FIG 3 Phylogenetic network with concatenated 16S rRNA, 23S rRNA, *groEL*, *rpoB*, and *dnaK* sequences (3,009 unambiguously aligned base pairs), including 71 *Coxiella*-like strains of ticks, 15 *C. burnetii* reference strains, and bacterial outgroups. (Adapted from reference 205.)

defined gene mutation obtained with the *Himar* transposon system inserted in the gene for FtsZ, a protein critical for cell division (212). However, at that time the absence of an axenic medium made the procedure very cumbersome, requiring 8 to 12 weeks to obtain mutants. The elaboration of the axenic medium ACCM2, allowing host cell-free growth of *C. burnetii*, has been a real breakthrough in this domain. Thanks to axenic culture, Omsland et al. reduced the time to obtain transformants to 16 days: 4 days for transformant recovery in ACCM2 and 12 days for colony development and expansion (41). Recently, Beare et al. reported two methods of targeted gene deletion in *C. burnetii* grown axenically. They used a Cre-*lox*-mediated or a loop-in/loop-out system for recombination and deletion (213). These two systems were used for the deletion of *dotA* and *dotB*, which are genes involved in the T4SS. Both strategies used a *sacB*-mediated sucrose counterselection to select for mutants (*sacB* produces an enzyme that converts sucrose to levans, which are toxic for most Gram-negative bacteria). Both systems were successful in producing *dotA* and *dotB* mutants, which presented a significant lack of intracellular replication owing to failure in the production of the intracellular vacuole. These new possibilities of targeted gene deletion will significantly improve the understanding of *C. burnetii* virulence mechanisms. Putative virulence and nonvirulence genes will be easily knocked out because of genetic transformation.

PATHOPHYSIOLOGY

Role of the Strain in Virulence

Primary infection. (i) Comparison of strain virulence in animal models. Since the 1960s, several animal models, including mice, guinea pigs, and anecdotally rabbits and nonhuman primates, have been used to describe *C. burnetii* pathogenicity (214–220). However, none of these models is able to mimic the disease observed in humans. Murine rodents are poorly susceptible to *C. burnetii* infection, and consequently a high dose of this bacterium is necessary to induce organ lesions. The A/J mouse strain was described as the strain most sensitive to *C. burnetii* infection, with a higher mortality rate than BALB/c and C57L/6 mice (221, 222). It has been considered the best mouse model to evaluate the immune response to *C. burnetii* infection and vaccine efficacy (221). BALB/c and C57BL/6 mice showed minimal and transient signs of infection and

no deaths. They were considered resistant to *C. burnetii* infection. In contrast, severe combined immunodeficiency (SCID) and nude mice present durable and severe signs of the disease and even die. The 50% lethal dose of *C. burnetii* Nine Mile strain in SCID mice was 10^8 lower than that in BALB/c mice, making SCID mice the model of choice to study the virulence of this bacterium (223). Clinical signs of discomfort, bacterial burden, and histological findings were stronger and occurred earlier in SCID mice than in A/J, BALB/c, and CB57BL/6 mice (86, 224). Nude mice or athymic mice are useful models for evaluating the role of cell-mediated immunity in the host defense against Q fever (225). Interleukin-10 (IL-10) transgenic mice, which constitutively overexpress IL-10 in their macrophage cells, are used to study persistent *C. burnetii* infection (226, 227). Guinea pigs are globally most susceptible to Q fever, which represents a life-threatening disease in these animals. They are used to study *C. burnetii* virulence, dissemination, and persistent infection (223, 228, 229). Rabbits were anecdotally used to study the ability of the Priscilla and Nine Mile strains to induce endocarditis (217). Finally, the nonhuman primate model of cynomolgus monkeys showed a *C. burnetii* infection very close to that observed in humans. These animals were used to study *C. burnetii* vaccine efficacy (219, 220, 224).

The pathogenicity and virulence of *C. burnetii* depend on the infected animal species, the route of infection, the *C. burnetii* strain, and the inoculum size (186). The first experiments were performed using intraperitoneal inoculation of *C. burnetii*, which led to a short incubation time and diffuse infection (230). Using intranasal injection and aerosolization routes, organ lesions were observed 7 days after infection and involved mainly the lungs, with lower systemic spread of bacteria. Since then, aerosolization of *C. burnetii*, which better mimics the natural route of infection, has become the reference method to induce *C. burnetii* infection in animals for pathogenicity studies (231–233).

The bacterium's virulence includes its capacity to induce clinical symptoms such as fever, splenomegaly, and death. Early studies have compared the pathogenicity of the Nine Mile phase I and the Priscilla phase I strains of *C. burnetii*, which present different phase I lipopolysaccharides (LPSs) (217). Intraperitoneal inoculation of *C. burnetii* in guinea pigs showed that an inoculum of fewer than 4 organisms of the Nine Mile phase I was sufficient to induce seroconversion and fever, whereas more than 10^5 bacteria of the Priscilla strain were necessary to induce fever (234). The lower virulence of the Priscilla strain in comparison to the Nine Mile strain was then confirmed by Kazar et al. in BALB/c mice and guinea pigs infected via the intraperitoneal route (235). Using aerosolization in guinea pigs and SCID mice to compare the pathogenicities of 8 *C. burnetii* strains from four phylogenetic groups, Russel-Lodrigue et al. confirmed that the Priscilla strain was less virulent than the Nine Mile strain, as evidenced by a delayed onset of symptoms, a slower progression of the disease, and a lower bacterial load in the spleens in animals infected with the former strain (223). In contrast to data obtained in humans, the Priscilla strain was not associated with a higher risk of endocarditis, probably because host factors such as preexisting valvulopathy were not considered in this work. However, these last results correlated with previous studies of Q fever endocarditis in rabbits, showing that the Priscilla strain did not induce more cardiac lesions than the Nine Mile strain (217, 236). Comparing the Nine Mile RSA493 strain to 8 other isolates (African RSA334, Ohio 314RSA270, MSU Goat Q177, P Q172, G Q212, S Q217, and Dugway 5J108-111), Russel-Lodrigue et al. showed that the Nine Mile strain was the most virulent (223). Nine Mile strain pathogenicity has been widely investigated in mouse models, but none have compared its virulence with those of *C. burnetii* epidemic strains. We recently compared the virulence of the *C. burnetii* French Guiana and Netherlands-like strains to the referent Nine Mile strain using SCID and BALB/c mice. The Netherlands-like strain was isolated in 1992 in Germany from a ewe placenta and displayed the same MST33 genotype as the one responsible for the Netherlands outbreaks (143). We first noted that SCID mice infected with the French Guiana strain presented more severe signs of infection, with up to 20% weight loss at day 28 postinfection, whereas symptoms appeared later with the Netherlands-like and the Nine Mile strains (237; C. Melenotte et al., presented at the ESCCAR International

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Congress on Rickettsia and Other Intracellular Bacteria, 13 to 16 June 2015, Lausanne, Switzerland). Spleen weights and bacterial loads were significantly higher and histological lesions appeared earlier in mice infected with the French Guiana and the Netherlands-like strains than in those infected with the Nine Mile strain. Finally, the serological response was exacerbated with the Guiana and the Netherlands-like strains, which corroborated data obtained in humans (238). This *in vivo* model highlights the higher virulence of the two epidemic strains, Guiana and Netherlands-like, than of the Nine Mile strain. The French Guiana strain was found to be the most virulent, followed by the Netherlands-like strain, which displayed intermediate virulence potential between those of the French Guinea and the Nine Mile strains.

(ii) Role of secretion systems in virulence. The sequenced genome of *C. burnetii* (Nine Mile RSA493) revealed the presence of several genes encoding adhesion, invasion, detoxification, and secretion system proteins (9). Interestingly, genes encoding components (IcmT, IcmS, and IcmK) of a type IV secretion system (T4SS), which are mechanistically related to the *Legionella* Dot/Icm apparatus (239), may contribute to the formation of the *C. burnetii*-containing vacuole (240, 241). The propagation of in axenic (host cell-free) culture (38, 41) has opened a technique for the genetic manipulation of this bacterium. The manipulation of an avirulent strain of *C. burnetii* and screening of the *C. burnetii* DNA library for genes encoding protein effectors have highlighted the fact that the *C. burnetii* Dot/Icm T4SS is involved in the establishment of a mature *C. burnetii*-containing vacuole and thus in intracellular multiplication of this pathogen (240, 241). However, such results could not be extrapolated to virulent strains. More recently, the importance of secretion systems in *C. burnetii* virulence was unraveled using the virulent *C. burnetii* 175 strain causing Q fever cases in Cayenne, French Guiana. The authors suggested that the virulence of the *C. burnetii* 175 strain is related to the loss of the type 1 secretion system (T1SS) encoded by the *hlyCABD* operon and thus to a genome reduction event (144).

Persistent infection. (i) LPS. The genetic diversity and virulence potential of *C. burnetii* strains are related to the expression of the lipopolysaccharide (LPS). *C. burnetii* displays LPS antigenic variations. The phase I infective form of *C. burnetii* is isolated from patients with Q fever. The avirulent form, known as phase II bacteria, is obtained following long-term *in vitro* propagation of *C. burnetii* in cell cultures (86). This leads to an irreversible modification of the LPS, with a progressive decrease in its molecular weight, up to a severely truncated LPS (rough form), owing to a genomic deletion (86). Lipid A of the LPSs from virulent and avirulent *C. burnetii* strains displays the same ionic species and fragmentation profiles, suggesting that it has a very similar structure in both types of strains (242). The major difference between the LPSs of virulent and avirulent *C. burnetii* strains resides in the core sugar, since the O antigen is missing in the LPS of the latter strains. The LPS of virulent strains (but not that of avirulent strains) contains sugars such as virenose, dihydrohydroxystreptose, and galactosamine uronyl-(1,6)-glucosamine (243). Interestingly, an intermediate-length LPS has been characterized at the surface of the Nile Mile Crazy strain (244), due to a large chromosomal deletion eliminating open reading frames involved in the biosynthesis of O-antigen sugars, including the rare sugar virenose (245).

(ii) Other virulence factors. Adhesion genes encode an RGD motif or proteins containing ankyrin repeats (9). Interestingly, the eukaryotic cell receptor for *C. burnetii* is an integrin $\alpha v\beta 3$, which interacts with the RGD motif (246). Genes encoding invasion proteins are similar to those engaged in cytoskeleton reorganization and uptake of *L. pneumophila* and enteropathogenic *Escherichia coli* by host cells. These genes probably play a role in the different levels of uptake of virulent and avirulent *C. burnetii* strains (246) and cytoskeleton reorganization observed during cell infection by *C. burnetii* (247). Detoxification genes encode superoxide dismutase, catalase, and acid phosphatase. These enzymes have been described as allowing *C. burnetii* to escape from the microbicidal activity of macrophages due to the detoxification of reactive oxygen intermediates produced by the host cells (248, 249). In addition, in the *C. burnetii* genome, a gene coding for a peptidyl-poly-*cis-trans*-isomerase has been identified,

similar to those expressed by *L. pneumophila*. It has been suggested that peptidyl-poly-*cis-trans*-isomerase may interfere with cytokine production and consequently with the replication of *C. burnetii* (250).

Role of the Host

Immunological response and phase variation. Humans develop primary *C. burnetii* infection that is symptomatic in fewer than half of cases. The primary infection almost always resolves without antibiotics, suggesting that the host immune response is sufficient to control *C. burnetii* infection (251). The host defense relies on systemic cell-mediated immunity involving innate and adaptive partners of the immune response. One of the main features of the immune response is the formation of a granuloma under the control of gamma interferon (IFN-γ). Granulomas are characterized by an accumulation of immune cells around a central open space and limited by a fibrin ring, which led to the definition of a doughnut granuloma. They are rich in macrophages with different levels of maturation, including epithelioid cells and multinucleated giant cells (252, 253). The presence of neutrophils in granulomas suggests that these cells are involved in the defense against *C. burnetii*, as recently demonstrated in neutrophil-depleted mice (254).

These granulomas have a microbicidal activity against *C. burnetii*, since they are paucibacillary during Q fever infection (251). The presence of neutrophils in Q fever granulomas suggests that these cells play a role in the defense against *C. burnetii*. Incubation of beads coated with *C. burnetii* phase I or II with mononuclear cells allowed us to investigate the mechanisms of granuloma formation in *C. burnetii* infection. First, phase II *C. burnetii* was less efficient than phase I microorganisms in inducing granuloma formation; second, monocytes were more critical than lymphocytes in granuloma formation; and third, granulomatous cells exhibited a transcriptional program that clustered with that of IFN-γ-stimulated macrophages, which are known to be a canonical model of microbicidal cells (255, 256).

The formation of granulomas in naive patients underlines the role of myeloid cells in the initial interaction of *C. burnetii* with innate immune cells. There is an exhaustive literature, including our contributions, which shows monocytes and macrophages to be major targets of *C. burnetii*. This bacterium binds to $\alpha v\beta 3$ integrin expressed by myeloid cells and induces their activation, as assessed by remodeling of the cytoskeleton, signal signaling activation, and production of a large cytokine panel (246, 251). There is also a certain level of polarization of monocytes and macrophages after they have encountered *C. burnetii*. Resting monocytes that allow bacterial survival without replication exhibit an M1-type program similar to that induced by IFN-γ. *C. burnetii* replicates in macrophages and induces an M2-related program similar to that induced by IL-4 or IL-10 (257).

Dendritic cells (DCs) are specialized in antigen presentation to T cells and have been more recently recognized as targets of *C. burnetii*. Initial studies revealed that phase I *C. burnetii* infects and blocks the maturation of human dendritic cells, in contrast to phase II *C. burnetii* (258). Recently, we compared the responses of myeloid DCs (mDCs) to a panel of intracellular pathogens and demonstrated that the blockade of mDC maturation was partial, since they were still able to induce T-cell proliferation. The transcriptomic analysis of *C. burnetii*-stimulated mDCs revealed subtle alterations in type I IFN signaling (259). We also showed that *C. burnetii* is able to activate plasmacytoid DCs (pDCs), inducing their maturation and the release of type I IFN, a property of the host response to virus. The presence of *C. burnetii* within pDCs in Q fever lymphomas may be indicate an original role of pDCs during Q fever.

Besides $\alpha v\beta 3$ integrin, the recognition of *C. burnetii* requires pattern recognition receptors, including Toll-like receptors (TLRs). Nevertheless, there are discordant data in the literature concerning their respective roles. We showed that TLR4 is involved in *C. burnetii*-stimulated cytoskeleton reorganization and the inflammatory response. However, it is not necessary for bacterial elimination *in vivo* (260). TLR2 plays a role in the type 1 immune response induced by phase II *C. burnetii*, and TL2-deficient macro-

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phages are permissive to bacteria (242). We found that TLR2 is required for granuloma formation, as is TLR4 (261). The nature of the LPS from *C. burnetii* may explain the discrepancies about the role of TLR in bacterium-cell interactions. The lipid A of *C. burnetii* LPS is tetra-acylated, whereas LPS from *Escherichia coli* is hexa-acylated. Phase I LPS from *C. burnetii* would be a poor immunogenic stimulus and functions as an antagonist of TLR4-dependent signaling in macrophages, in contrast to LPS from *E. coli* (242). The use of a mouse model does not allow identification of this TLR4 antagonist property of *C. burnetii* LPS. Recently, it has been reported that humans with polymorphisms in TLR1 and NOD2 have reduced production of cytokines after infection with the *C. burnetii* Nine Mile and Dutch strains, and those with a polymorphism in TLR6 have an alteration of cytokine production only with the Dutch strain (262). Recently, it was reported that TLR10 exerts an inhibitory effect on cytokine production by *C. burnetii*-stimulated mononuclear cells; the polymorphisms of TLR10 described in Q fever patients were not associated with a specific clinical expression or prognosis (263).

The adaptive immune system is required to cure *C. burnetii* infection. The use of murine models such as SCID mice (264) and the follow-up of patients with immunosuppression (86) strengthen this statement. There is an unequal efficiency of the two arms of the adaptive immune response, T cells and antibodies.

T cells are necessary to control the infection, since nude and SCID mice are highly sensitive to *C. burnetii* infection (225, 264), and the reconstitution of SCID mice with $CD4^+$ or $CD8^+$ T cells is sufficient to restore protective immunity. It seems that $CD8^+$ T cells are more efficient in controlling *C. burnetii* infection than $CD4^+$ T cells (265). Recently, we found that central memory $CD8^+$ T cells are increased, whereas naive $CD8^+$ T cells are decreased, in patients with Q fever endocarditis (266). We also showed that the expression of PDL-1 is increased in patients with acute Q fever, and this would prevent the expansion of memory T cells (266). Such an imbalance of circulating lymphoid cells may play a major role in the chronic progression of Q fever.

It has been established for a long time that the polarization of the T-cell response toward the Th1 phenotype, i.e., the ability to produce IFN-γ, was the paradigm of the protective response against intracellular pathogens. Indeed, it has been shown that virulent *C. burnetii* induces a Th1 protective immune response, in contrast to avirulent variants (267). Some epitopes of *C. burnetii*, identified by bioinformatics, are able to stimulate Th1 T cells (268). There is converging evidence that IFN-γ makes macrophages resistant to *C. burnetii*. First, mice with knockouts (KO) in the IFN-γ gene are highly susceptible to *C. burnetii* infection (269). Second, we showed that IFN-γ induces a microbicidal response against *C. burnetii* in macrophages (270). The mechanisms used by IFN-γ to control *C. burnetii* infection are diverse and vary with the type of host. The microbicidal effect of IFN-γ does not depend on reactive oxygen intermediates, and Q fever is no more frequent in patients with chronic granulomatous disease (270, 271). The role of reactive nitrogen intermediates is debated, and if they contribute to the IFN-γ effect, this is restricted to murine models (242, 270, 272, 273). IFN-γ induces a microbicidal program directed at *C. burnetii* via oxygen-independent mechanisms, including phagosome maturation (274), apoptosis (270), production of cytokines such as tumor necrosis factor (TNF) (275), and regulation of nutrients such as iron (251). Taking the data together, a reductionist point of view about the role of Th1 cells and IFN-γ had prevailed in earlier years. The primary infection, with *C. burnetii* progressing to cure or the immune response to vaccination, was considered to correspond to a Th1 immune response with IFN-γ production, while both were considered defective in patients with persistent *C. burnetii* infection (276, 277). However, Dutch teams have demonstrated that, in contrast to what had been generally assumed, the production of IFN-γ is not defective in persistent *C. burnetii* infection (278, 279). The production of IFN-γ is used as a specific biomarker of chronic Q fever and, like IL-2, it is a good marker of antibiotic treatment efficacy (233, 280). In addition, these patients respond to IFN-γ by upregulating genes downstream of the IFN-γ-receptor and producing neopterin (T. Schoffelen, J. Textoris, C. P. Bleeker-Rovers, A. Ben Amara, J. W. M. van der Meer, M. G. Netea, J.-L. Mege, M. van Deuren, and E. van de Vosse, submitted for publication). The

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fact that genetic polymorphisms in IL12B are associated with the development of Q fever (Schoffelen et al., submitted) emphasizes the fact that persistent *C. burnetii* infection does not result from a deficiency in Th1 polarization.

If the persistence of *C. burnetii* is not the consequence of a defective type 1 immune response, several hypotheses can be considered. There is an immunosuppressive context in the persistent progression of Q fever. There are changes in circulating immune cells, including depressed $CD4^+$ T cells (281), naive $CD8^+$ T cells (266), nonclassical monocyte subsets (282), $CD56^{dim}$ NK cells, and increased naive $CD8^+$ T cells (266) and regulatory T cells (283). There is also an exacerbated inflammatory response consisting of high levels of TNF, IL-1, IL-6, CD23, neopterin, and chemokines (251, 275, 284, 285). It is likely that this uncontrolled inflammatory reaction may contribute to the pathogenesis of Q fever. Also, the persistence of *C. burnetii* is associated with the upregulation of immunoregulatory mediators such as prostaglandin E_2 (276) and IL-10 (286, 287).

The second arm of the adaptive immune response, antibodies, has been considered dispensable for host protection against *C. burnetii* infection. Animal models and human infection are characterized by the production of specific antibodies. Initial studies showed that passive transfer of antibodies protected guinea pigs from a subsequent challenge with *C. burnetii* (288). Passive immunization of naive mice with antibodies isolated from vaccinated mice provided full protection (267). However, immune sera or B cells from *C. burnetii*-challenged mice do not confer protection against *C. burnetii* when transferred to SCID mice (267). High antibody titers directed to phase I and phase II antigens are found in persistent *C. burnetii* infection and are not effective for *C. burnetii* clearance. Moreover, it is likely that these specific antibodies have deleterious effects in Q fever patients. They can contribute to tissue lesions via the formation of immune complexes (289). They can also regulate the activity of cells expressing receptors for immunoglobulins, but that point remains debated. We showed that *C. burnetii* opsonized with specific immunoglobulins from Q fever patients is more readily internalized, reaches multibacillary vacuoles, and stimulates the production of IL-10 (290). Other groups did not find an immunosuppressive effect of opsonization but found rather that it could favor DC maturation and cytokine production (291). Finally, antibody formation during *C. burnetii* infection can target host components, and autoimmunity has been associated with acute Q fever (292–294). During primary *C. burnetii* infection, anticardiolipin antibodies are usually detected, and high levels of IgG anti-cardiolipin antibodies have been associated with rapid progression to *C. burnetii* endocarditis (295).

In conclusion, the control of *C. burnetii* primary infection is obtained via the induction of a full cell-mediated immune response, in which the Th1 response and IFN-γ production are the most important, while antibodies are dispensable. This strong Th1 response may cause autoimmune disorders. The progression to *C. burnetii* persistent infection reflects failure of the Th1 response and results from a combination of intrinsic and extrinsic parameters, in which IL-10 plays a significant role.

Determinants of the intracellular persistence of *C. burnetii*. In contrast to the case for human mononuclear phagocytes, cumulative evidence suggests that *C. burnetii* vacuoles mature through the endolysosomal cascade to a phagolysosome in nonprofessional phagocytes. However, this is overly simplified, because *C. burnetii* modifies the phagosome to create a vacuole with fusion with other endolysosomal and autophagic compartments and cargo from secretory pathways (296).

In human mononuclear phagocytes, the intracellular trafficking of *C. burnetii* has been well described (274). After phagocytosis, virulent and avirulent *C. burnetii* organisms are localized within early phagosomes harboring early endosome antigen 1 (EEA-1) and the small GTPase rab5. The early phagosome is then converted into a late phagosome, characterized by lysosome-associated membrane protein 1 (LAMP-1), LAMP-2, and LAMP-3, the mannose-6-phosphate receptor (M6PR), and vacuolar proton ATPases responsible for the acidic pH (pH 4.5) of the compartment containing virulent or avirulent *C. burnetii*. The phagosome hiding avirulent *C. burnetii* acquires the small

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rab7, which is required for phagolysosome transition (297–299), and the lysosomal enzyme cathepsin D (274). In this compartment, avirulent bacteria are destroyed. Interestingly, virulent *C. burnetii* blocks the conversion of the late phagosome into a phagolysosome by inhibiting the recruitment of rab7 and cathepsin D (274, 300), avoiding its degradation in the phagolysosome. Part of the mechanisms used by virulent *C. burnetii* to hijack the phagosome maturation has been elucidated (301, 302). Indeed, it has been demonstrated that variations in LPS determine the intracellular localization of *C. burnetii*. Avirulent *C. burnetii* LPS stimulates host p38α–mitogen-activated protein kinase (MAPK) signaling, which is required for the trafficking of bacterium-containing vacuoles to phagolysosomes for their destruction. In contrast, *C. burnetii* LPS does not. The defect in *C. burnetii* targeting to degradative phagolysosomes involves an antagonistic engagement of Toll-like receptor 4 (TLR4) by *C. burnetii* LPS, lack of p38α-MAPK-driven phosphorylation, and blockade of recruitment of the homotypic fusion and protein-sorting (HOPS) complex component vacuolar protein sorting 41 (Vps41) to *C. burnetii* LPS-containing vesicles. Thus, p38α-MAPK and its cross talk with Vps41 play a central role in trafficking *C. burnetii* to phagolysosomes (301). In addition, it has been recently demonstrated that *C. burnetii* avoids p38α-MAPK activation by the disruption of the association of TLR2 and TLR4 via an actin cytoskeleton reorganization induced by LPS (302). Several other factors, such as opsonization (290) and cytokine production (303), may greatly contribute to the establishment of the *C. burnetii* replicative vacuole, but the mechanisms involved remain unclear. Opsonization of *C. burnetii* with specific antibodies produced during persistent Q fever seems to prevent phagosome conversion, because large parasitophorous vacuoles containing *C. burnetii* but not expressing cathepsin D are formed (290). Cytokines have been shown to modulate phagosome maturation (303). In case of *C. burnetii* infection, several data suggest that IL-10 could contribute to the establishment of *C. burnetii* replicative phagosomes. The neutralization of IL-10 from monocytes of patients with persistent *C. burnetii* infection increases the killing of *C. burnetii* and rescues phagolysosome fusion similarly to what is observed in cured patients. In contrast, adding IL-10 to monocytes from patients with cured *C. burnetii* endocarditis avoids the killing of *C. burnetii* and inhibits phagolysosome biogenesis (300).

Host susceptibility factors. Epidemiological studies have shown that Q fever symptoms are more frequent in men than in women (male/female ratio, 2.5), while both are similarly exposed to this pathogen, as evidenced by similar seroprevalence rates (304, 305). Recently, the follow-up of patients vaccinated during the Q fever outbreak in the Netherlands revealed higher rates of adverse effects in women than in men (306). The use of murine models of infection revealed the role of sex hormones in the predisposition for infection in men. Hence, female C57/BL6 mice are more resistant than males to *C. burnetii* infection; this property is lost after ovariectomy and is restored by the administration of 17β-estradiol (307). The study of gene expression signatures in mice infected with *C. burnetii* with and without castration shows the importance of sex-related genes (86% of genes differentially modulated in males and females) and the role of sex hormones, which account for 60% of modulated genes (308). The functional annotation of modulated genes enabled the identification of different clusters in males and females. The enrichment in clusters containing genes associated with the inflammatory response may account for the inflammatory profile of men with Q fever. Among the clusters enriched in infected females, the circadian rhythm pathway (consisting of positive molecules such as Clock and Arntl and negative molecules such as Per) is still not understood and may reveal some features of a microbicidal response to *C. burnetii*. Data for humans are rare. It has been shown that the expression of Per2 is increased in men with acute Q fever (309).

(i) Age. Age is a risk factor for *C. burnetii* infection. Symptomatic Q fever is more frequent in individuals older than 15 years (310). The immunity of adults to *C. burnetii* accounts for the adult response to vaccine. During the vaccination campaign with Q-vax vaccine in the Netherlands, the best cellular and humoral responses to the

antigen as well as protection against the infection were reported in young adults, who also suffered most frequently from local adverse effects (306).

In 14-month-old mice (corresponding to adult humans), the bacterial burden and the number of granulomas in tissues were higher than those in 1-month-old mice (307). Elderly patients develop an immune response to *C. burnetii* that reflects the level of immunosenescence. Patients at risk of persistent Q fever who are older than 75 years are lower producers of IFN-γ and specific antibodies than younger patients (311). The use of immunosuppressive drugs is likely a confounding factor.

(ii) Pregnancy. The pathophysiology of Q fever during pregnancy is poorly understood, especially in humans. The infection of goats with *C. burnetii* provided some mechanistic hypotheses. After inoculation of pregnant goats, the primary targets are the trophoblasts of the allantochorion, while trophoblasts covering cotyledonary villi are not infected. The infection leads to the production of specific antibodies that persist for several months after delivery. There is an inflammatory reaction varying from mild infiltration of mononuclear cells to necrotic lesions (90). There is also a cellular response during and after parturition. IL-10 is upregulated during pregnancy, whereas TNF and IL-1 are increased after parturition (312). In women, there is some evidence that the immune response is silenced and antibodies are produced in response to infection (313). This might be related to IL-10 production, which may account for the increased risk of endocarditis after delivery. In human trophoblasts infected *in vitro* with *C. burnetii*, the bacteria replicate within vacuoles that express lysosomal markers, in contrast to myeloid cells. In addition, the interaction of *C. burnetii* with trophoblasts does not induce an inflammatory program, accounting for the relative immune silence observed during Q fever infection in pregnant women (314). This is strengthened by a recent study in which we showed that *C. burnetii* is unable to activate decidual DCs (315). It is likely that alterations of the immune response during pregnancy correspond mainly to the production of IL-10 and the silencing of DCs, which favors bacterial growth (312, 314).

(iii) Genetic factors. Preexisting valvulopathy enhances the risk of endocarditis in patients with *C. burnetii* infection. The lack of vegetation and inflammation suggests a mechanism distinct from the colonization of cardiac valves found in usual infectious endocarditis. This suggests that factors related to the cardiac valve context, including the immune context, are involved. The study of cardiac valves and circulating mononuclear cells from patients with degenerative valvulopathy using microarray technology revealed the enrichment of the inflammatory program (316, 317); this creates the condition for the binding of inflammatory leukocytes, infected or not with *C. burnetii*. These findings are supported by the detection of activated lymphocytes and macrophages infiltrating bicuspid and tricuspid calcified stenosis (318). The study of lymphocytic populations revealed the important role of $CD8^+$ T cells with a memory-effector phenotype and oligoclonal repertoire, while the role of memory-effector $CD4^+$ T cells was less significant (319). *C. burnetii* endocarditis is more frequently observed in patients with bicuspid aortic valves (BAV) (320). BAV is the most frequent congenital heart disorder and has a sizeable heritable component, with some genes specifically associated with this entity (321). The analysis of genes expressed in BAV compared with tricuspid aortic valve controls showed the enrichment of the latter, with genes associated with inflammation and the immune response, compared with the former, in which genes involved in the development are overexpressed. This poor inflammatory context of BAV may favor the colonization of valves with infected cells. The mechanism of Q fever endocarditis in patients with BAV remains hypothetical. Although mechanical conditions such as increased flow turbulence due to inappropriate opening are likely, we hypothesize that apoptosis may be involved. Indeed, BAV is associated with increased apoptosis of vascular smooth muscle cells (322).

Role of IL-10

IL-10 properties. Interleukin 10 (IL-10) is a class 2 cytokine produced by macrophages, monocytes, dendritic cells (DCs), lymphocytes, B cells, mast cells, eosinophils,

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and $CD4^+$ T cells. The main functions of IL-10 are immunosuppressive and anti-inflammatory, influencing both innate and adaptive responses. Hence, IL-10 contributes to maintaining appropriate conditions for microbe survival and persistent infection. During the innate immunity response to microbes, IL-10 downmodulates the production of inflammatory mediators and promotes the expression of anti-inflammatory mediators, including regulatory receptors such as the IL-10 receptor by myeloid cells. IL-10 has a strong potential for inhibiting the microbicidal activity of macrophages and the antigen-presenting activity of dendritic cells. On the other hand, IL-10 enhances its own production by $CD4^+$ regulatory T cells. The anti-inflammatory properties of IL-10 were first demonstrated in IL-10-deficient mice, which developed chronic bowel disease, probably secondary to an inappropriate and aberrant immune response to bacterial antigens. During the adaptive response, IL-10 downregulates the production of Th1 cytokines, such as IL-2, IL-3, IFN-γ, and major histocompatibility complex class II (MHC II), and costimulatory molecule expression and interferes with T-cell polarization. Thus, IL-10 promotes the proliferation and differentiation of B cells (323, 324). Animal models have been used to exhaustively describe the influence of IL-10 on host susceptibility during primary infection (323). The reduction of IL-10 increases the resistance to infection, whereas IL-10 overproduction increases host susceptibility to bacteria and parasites (323). Interleukin-10 levels are determinant in the clearance or the persistence of pathogens infecting humans (324, 325). Hence, IL-10 is associated with tuberculosis (TB) reactivation, *Bartonella henselae* persistent infection, and *Bartonella quintana* chronic asymptomatic bacteremia (326, 327). In Q fever patients, *C. burnetii* is able to persist in macrophages, to escape the immune system, and to induce persistent infection; this persistence has been associated with sustained production of IL-10 (284, 300) (see below).

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Role of IL-10 in endocarditis. *C. burnetii* endocarditis lesions exhibit fibrosis, calcification, slight inflammation and vascularization, and small or absent cardiac vegetation, probably due to the intracellular nature of the bacterium (328). The persistence of *C. burnetii* in the infected host is characteristic of the progression to endocarditis. This statement is the consequence of several observations during the past years. First, IL-10 is secreted by mononuclear cells in patients with *C. burnetii* primary infection and valvulopathy at risk of developing endocarditis (286, 287). Second, IL-10 is important for the persistence of *C. burnetii*, since it interferes with the microbicidal program of host cells via the downmodulation of TNF production (300). The effect of IL-10 is not a result of nonspecific immunosuppression of macrophages, since other immunoregulatory cytokines failed to induce *C. burnetii* replication (227). Third, the impairment of macrophage microbicidal activity in patients with Q fever endocarditis is under the control of IL-10 (300). Fourth, the use of mice overexpressing IL-10 in the myeloid compartment reproduces most of the features of Q fever endocarditis, including bacterial persistence, lack of granuloma formation, and overproduction of specific antibodies (226). However, IL-10 by itself is not sufficient to cause endocarditis. Fifth, IL-10 is produced during Q fever by monocytes, by dendritic cells, and in response to anti-*C. burnetii* antibodies. When the latter are used as opsonizing antibodies, they favor the formation of multibacillary vacuoles within the macrophages and the secretion of IL-10 (290). This amplification loop may be critical in the pathophysiology of *C. burnetii* endocarditis, since IL-10 favors the production of antibodies, and specific antibodies via the recognition of phospholipid determinants are likely involved in the progression to endocarditis. The presence of IL-10 may also explain the poor inflammatory phenotype of *C. burnetii* endocarditis. We hypothesized that the phagocytosis of apoptotic leukocytes known to lead to IL-10 release is involved in the formation of endocarditis. Valvulopathy is associated with altered fluid shear stress and increased circulation of cells, including leukocytes (257). The uptake of leukocytes by monocytes and macrophages stimulates the replication of *C. burnetii* and the production of IL-10. It is likely that the immune context will determine the development of endocarditis: if patients with valvulopathy are able to exhibit a Th1-like immune response, the effects of apoptosis and Il-10 are prevented; in contrast, patients unable to develop a Th1-like response would have an

increased risk of *C. burnetii* endocarditis (251). This hypothesis will require confirmation in an animal model.

Role of IL-10 in lymphoma. IL-10 is known to play a role in B-cell lymphoma genesis by promoting B-cell proliferation and by compromising the function of immune cells, which then become unable to kill tumor cells (329, 330). High levels of IL-10 are observed in patients with non-Hodgkin lymphoma and are associated with a poor prognosis (331, 332). IL-10 was also used as a prognostic and therapeutic marker in patients with diffuse large B-cell lymphoma (DLBCL) (330, 331, 333). It was also proposed as a new therapeutic target in DLBCL (333). *C. burnetii* has recently been reported as being linked to B-cell non-Hodgkin lymphoma (334). Patients with Q fever presented an excess risk of DLBCL and follicular lymphoma (FL) compared to the general population. The direct evidence for the role of *C. burnetii* in lymphomas was provided by the study of tumoral biopsy specimens using fluorescence *in situ* hybridization; viable *C. burnetii* cells were present within macrophages and plasmacytoid dendritic cells (pDCs) but not in B cells. Furthermore, patients with Q fever and lymphoma presented an overproduction of IL-10 compared to that in patients with Q fever lymphadenitis or with acute Q fever but no valvulopathy. High levels of IL-10 were also observed in patients with *C. burnetii* lymphadenitis, in which *C. burnetii* was identified in lymph node macrophages but not in pDCs (334). By infecting macrophages and pDCs and by inducing overexpression of IL-10, *C. burnetii* might be responsible for an immune impairment that promotes both *C. burnetii* replication within the tumoral microenvironment and tumoral growth (334, 335).

CURRENT TOOLS FOR LABORATORY DIAGNOSIS

Because *C. burnetii* does not grow with the use of standard routine laboratory culture techniques, specific indirect diagnostic tools have been mainly used for diagnosis. Consequently, serology is still the most common method for testing for *C. burnetii* infection. Currently, detection of *C. burnetii* DNA by qPCR in various clinical samples (including blood, cardiac valves, or other surgical tissue biopsy specimens) is also available. It has the advantage of detecting *C. burnetii* before seroconversion in patients with primary infection. Culture can be performed by reference laboratories on the same clinical samples, but this requires a biosafety level 3 (BSL3) laboratory. Finally, pathological analysis of infected tissue samples, after immunohistochemistry staining, is an interesting tool for diagnosis when these samples are available. Improvement of the sensitivity of the main diagnostic techniques has been the principal objective in recent years.

Serology

General principles. In the presence of symptoms suggestive of *C. burnetii* infection, serology is the first-line diagnostic technique. The immune response induces the production of anti-phase II and anti-phase I antibodies (42). *C. burnetii* phase II antigen is obtained after several passages in cell cultures or eggs, and anti-phase II antibodies are predominant during primary infection (336). *C. burnetii* phase I antigen is obtained from the spleens of infected mice, and anti-phase I antibodies are associated with persistent infection (336). The phase II antibodies are detectable 7 to 15 days after the onset of clinical symptoms and decrease thereafter within 3 to 6 months (42). The diagnosis of primary infection can be made by detection of a 4-fold increase in phase II IgG or IgM antibodies between two serum samples taken 3 to 6 weeks apart (42). Antibodies are detectable by the third week after infection in 90% of patients (336). For that reason, two serum samples (one from the acute phase and one from the convalescent phase) should be analyzed. Cutoffs for a positive serological titer can vary between countries. Generally, titers of phase II IgG of $\geq$200 and/or IgM of $\geq$50 are considered significant for the diagnosis of primary Q fever infection (336, 337), and phase II IgG titers tend to be higher than phase I IgG titers during primary infection (42, 86). Independently of the symptomatology, residual IgG antibody titers may be detectable for years and even for life (338). Wielders et al. have shown that early treatment

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of primary infection (before an antibody response) did not influence the subsequent IgG II response (339).

Elevated phase I IgG titers (IgG I titer of ≥1:800) are associated with persistent Q fever. Higher phase I IgG titers correlate with a higher positive predictive value (PPV) for the diagnosis of *C. burnetii* endocarditis: a PPV of 37% was found for IgG I titers of ≥1:800, and this reached 75% for IgG I titers of ≥1:6,400 in a study from our reference center (340). For that reason, investigation for persistent infection should be performed in the case of persistent high levels of phase I antibodies 6 months after completion of treatment (see Management Strategy for Patients with *C. burnetii* Infection section and Clinical Aspects section below). However, cases of Q fever endocarditis with low antibody titers have been reported. In a series of 125 patients with cardiovascular infection who underwent surgery, we found 4 patients with a definite cardiovascular infection with positive culture and/or PCR on valvular biopsy, three of whom had a phase I IgG titer at 1:400 and one of whom had a phase I IgG titer at 1:200 (341).

Serology methods. Indirect immunofluorescence assay (IFA) is the reference method, but the complement fixation test (CFT) and ELISA are also used. Others techniques exist, such as Western blotting, dot immunoblotting, radioimmunoassay, microagglutination, and the indirect hemolysis test, but they remain anecdotal. To date, only IFA, CFT, and ELISA are commercially available. The advantage of ELISA is that it is easy to perform, interpretation is less subjective than for IFA and CFT, and automation is possible. This method is mentioned in the CDC case definition of acute and chronic Q fever (42). The specificity, sensitivity, and positive predictive value vary according to the technique and the antigen used. Wegdam-Blans et al. recently compared the performance of commercially available ELISA, CFT, and IFA. For the diagnosis of primary infection, the authors used serum samples from patients with a positive qPCR on blood (342). The most sensitive technique was IFA for detecting IgM antibodies at an early phase of infection and after 12 months of follow-up. Regarding IgG, IFA was more frequently positive than ELISA and CFT (100%, 95.2%, and 96.8%, respectively), but the difference was not statistically significant (342). Regarding phase I antibodies in patients with persistent *C. burnetii* infection at the time of diagnosis, the sensitivities of CFT and ELISA were 83% and 93.9%, respectively, but the difference was not statistically significant (343). Overall, using CFT, 16.3% of patients with phase I IgG were undetected. Also, a poor correlation was observed between antibody kinetics in ELISA, CFT, and IFA. Therefore, the sensitivity of CFT appears to be too low to be recommended for diagnosis and follow-up of *C. burnetii* persistent infection.

Most reference laboratories have developed their own in-house immunofluorescence assay. In our center, screening is performed with phase II antigen on serum diluted at 1:50 and 1:100 to detect total immunoglobulins (IgT) directed against *C. burnetii* antigens (344). For all positive screenings with IgT titers of ≥1:100, quantification detection of antibodies for the subclasses IgG, IgM, and IgA for both phase I and phase II is performed. The titration of IgM and IgA is performed after removal of IgG using a rheumatoid factor absorbent to eliminate false-positive results due to interference with this protein. Moreover, the sera are diluted in phosphate-buffered saline with 3% nonfat powdered milk to saturate the antigenic site and avoid a nonspecific fixation of antibodies. Sensitivity was assessed at 58.4% and specificity at 100% (344). For sera with titers inferior to these cutoffs, the serology should be repeated within 10 to 15 days to confirm or rule out the diagnosis.

One study compared Q fever serological results from different reference centers from three countries (United Kingdom, France, and Australia) (345). The concordance between the three centers in microimmunofluorescence interpretation was only 35%. France and the UK had the lowest concordance, and the UK and Australia had the highest. This reflects the fact that serology is not the most perfect tool, because of high subjective variability of interpretation. Cross-reactions with *Legionella micdadei* and *Bartonella* have been described with IFA (346, 347). Musso and Raoult found that 34.5% of patients with primary Q fever presented significant antibody titers for *L. micdadei* (346). La Scola and Raoult observed that sera from 50% of patients with Q fever

displayed cross-reactivity with *Bartonella* (347). However, in most cases, a low level of cross-reactions was observed, and antibody titers were higher for *C. burnetii*, so that generally there was no problem in the interpretation of results.

Recently, a new automated epifluorescence assay was developed by InoDiag (Signes, France) for Q fever serological diagnosis. Except for the deposition of the serum, all subsequent steps have been automated. The performance of this technique was recently compared with that of a gold standard microimmunofluorescence technique. It showed heterogenous performances, with a low sensitivity for primary infection due to low reactivity of phase II antigens but an excellent performance for persistent infection, with 100% sensitivity in the detection of phase I antigens (348).

Molecular Detection

Several PCR-based assays have been developed for the detection of *C. burnetii* in clinical samples. The first standard PCR systems targeted sequences of different types of plasmids (349), the 16S-23S RNA, the superoxide dismutase gene, the *com1* gene or the IS*1111* repetitive elements in human or animal samples (56, 350–352). The detection limits of these different methods ranged from 10 to 10^2 bacteria. Also, nested PCR systems have been proposed, but these methods lack specificity (353, 354). Real-time PCR or quantitative PCR (qPCR) is a less time-consuming technique than PCR and has the advantage of quantifying the amount of bacteria in clinical samples. Thus, this method has become the most frequently used PCR system for diagnosis. The qPCR system targeting IS*1111* (a repetitive element which is present in about 20 copies in the *C. burnetii* Nine Mile genome) is the most sensitive (124, 355, 356). This qPCR can detect the bacterium in the sera of patients within the first 2 weeks of infection, when serology is not yet positive. It also allows detection of *C. burnetii* DNA in the blood of patients with persistent *C. burnetii* infection (357). In the Netherlands, Schneeberger et al. found *C. burnetii* DNA in 10% of seronegative samples from patients with signs of primary infection, confirming the usefulness of this method in the first 2 weeks of infection (358). In another study from the Netherlands, this assay displayed sensitivity, specificity, PPV, and negative predictive value (NPV) of 92.2%, 98.9%, 99.2%, and 89.8%, respectively, during the outbreak (359). In that study, a high DNA load during primary infection was associated with progression to persistent infection. Tilburg et al. assessed the interlaboratory concordance of IS*1111* qPCR according to the assay and a DNA extraction method used in seven laboratories across the Netherlands (360). They found that multiple combinations of DNA extraction kits and qPCR assays gave equivalent results for Q fever diagnosis. In Switzerland, a qPCR system targeting the *ompA* gene has been used for 7 years for detection of *C. burnetii* in clinical samples (361). The sensitivity was 88% for valvular samples, 69% for blood samples, and 50% for urine samples. In our laboratory, another qPCR system targeting IS*30A* repetitive elements displayed a lower sensitivity than IS*1111* qPCR (124).

Recently, we improved the sensitivity of the qPCR test targeting the *IS1111* gene by concentrating DNA extracted from clinical samples by lyophilization (362). The detection limit of *C. burnetii* DNA was 100-fold lower in lyophilized sera (1 bacterium/ml) than in nonlyophilized sera (10^2 bacteria/ml). This strategy was tested in 73 sera from patients with primary *C. burnetii* infection and 10 sera from endocarditis patients, in whom the IS*1111* qPCR performed under the usual conditions remained negative. In patients presenting with primary Q fever, we observed qPCR sensitivity gains of 44% for the seronegative sera and 30% for early seropositive sera after lyophilization. The sensitivity of qPCR was also higher in sera from patients with endocarditis, of whom 8/10 (80%) were positive after lyophilization.

Culture

The isolation of *C. burnetii* can be achieved from a wide range of clinical samples, including old samples if they have been stored at −80°C before cultivation. The shell vial technique is still the most frequently used method (363). A sample of 1 ml of the clinical specimen is inoculated on HEL cell monolayers in shell vials. The shell vials are

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then centrifuged (700 × *g* at 20°C) for 1 h. Centrifugation allows better attachment and penetration of *C. burnetii* inside cells. Infected cells are then incubated at 37°C in a 5% CO_2-enriched atmosphere for 5 to 7 days. Gimenez or immunofluorescence staining is used for detection of the bacterium inside cells. Lockhart et al. have compared four different cell lines for the isolation of *C. burnetii* using two different isolates, the Henzerling and Arandale strains (364). For the Henzerling strain, DH82 cells were the most sensitive, while for the Arandale isolate, Vero cells showed the highest sensitivity. The L929 and XTC cell lines were less suitable for culture of *C. burnetii*.

Recently, the first isolation of *C. burnetii* in axenic medium from clinical samples was reported using ACCM2 (170). A sample of the heart valve from a patient with *C. burnetii* endocarditis was incubated in 20 ml of ACCM2, and growth was observed after 6 to 8 days of incubation. Inoculation of an ACCM2 agar plate with a sample of the culture-positive liquid ACCM2 yielded several colonies at day 5 (170). This new possibility could significantly facilitate the routine cultivation of *C. burnetii* from clinical samples.

Pathology and Immunohistochemistry

The immune reaction provoked in various organs by *C. burnetii* can be unraveled by pathological analysis of tissue samples after fixation and paraffin embedding. During primary infection, a typical fibrin-ring granuloma with a "doughnut" aspect can be observed on hepatic biopsy specimens (365). These granulomas have also been observed in the bone marrow (366–369). During persistent infection, pathological analysis of cardiac valves and vascular tissue can also be informative. In patients with *C. burnetii* endocarditis, histological analysis can reveal significant fibrosis, calcifications, slight inflammation and vascularization, and little or no vegetation (328). These features illustrate a slow "degenerative-like" infectious process. Immunohistochemical (IHC) detection is a more specific tool that can aid detection of *C. burnetii* in tissues. It uses a monoclonal antibody with an immunoperoxidase-based method (328). Lepidi et al. also developed a method called "autoimmunochemistry," using antibodies produced from the patient's own serum (370). IHC was used for detection of *C. burnetii* in aortic graft samples and hepatic and valvular biopsy specimens (328, 371, 372).

New Tools

Immuno-PCR. Immuno-PCR is an interesting method, combining the amplification power of PCR with the specificity and versatility of ELISA, allowing an improvement in sensitivity. We tested this method on a collection of serum samples from Q fever patients (373). Immuno-PCR had significantly better sensitivity than ELISA and IFA (90% versus 35% and 25%, respectively) in sera collected during the first 2 weeks after the onset of symptoms (373). Its specificity was evaluated at 92%.

IFN-γ and IL-2 detection. The detection of *C. burnetii*-specific gamma interferon (IFN-γ) production has been proposed as a new diagnostic tool. Schoffelen et al. have compared the performance of this method with those of classic IFA and skin tests for Q fever (278). The IFN-γ production assay is performed after *in vitro* stimulation of whole blood with antigens from the Q-vax vaccine or the inactivated Nine Mile strain. The measurement of IFN-γ production is then performed using ELISA. IFN-γ production was higher in seropositive and skin test-positive patients than in negative ones. The sensitivity and specificity (87% and 90.2%, respectively) were similar to those for the association of serology and skin test. In another study, the same authors evaluated the usefulness of this method in differentiating between past and persistent *C. burnetii* infection. IFN-γ production was significantly higher in patients with persistent infection than in controls, but overlapping values existed. Assay of a second cytokine, IL-2, revealed significantly higher values in controls than in patients with persistent infection. As a consequence, an IFN-γ/IL-2 ratio of >11 had a sensitivity and specificity of 79% and 97%, respectively, for the diagnosis of persistent infection (233). In a recent study, this ratio was proposed for monitoring treatment of persistent *C. burnetii* infection (233). It decreased significantly when patients had a successful

treatment outcome (233). Thus, an IFN-γ/IL-2 ratio of >11 may be an interesting additional marker to monitor the progress of patients with persistent infection.

CLINICAL ASPECTS

Chronic Q Fever: from Historical Background to Recent Controversy

Historical background. In 1937, Edward Holbrook Derrick reported the first clinical description of a *C. burnetii* infection when investigating an outbreak of febrile illnesses among slaughterhouse workers in Brisbane, Queensland, Australia (374). He proposed the name of "Q fever" for "query fever," until further knowledge could allow a better name. In the following years, the first U.S. case of human Q fever, in a member of the NIH staff who was cultivating an infectious agent isolated by Cox in Guinea pigs AQ: O inoculated with a tick sample, was reported (375). Several outbreaks in different parts of the world were then reported, including in the Americas in the 1940s (at the NIH, in California, and in Panama) (376–378) and in Europe among allied troops during World War II (in northern Italy and the Balkans) (379). In these early reports, "Q fever" was considered to be an acute disease causing outbreaks of fever and pneumonia.

In 1949, Beck and Bell were the first to note that: "several persons have been found with a chronic febrile illness dating back to a proved attack of Q fever" (378). Some years later, Marmion et al. described what they called "a subacute Rickettsial endocarditis," which was a negative blood culture endocarditis with high levels of Q fever phase I antibodies; isolation of the bacterium was obtained in guinea pigs from an aortic valve sample (380, 381). In these reports, the term "chronic Q fever" was coined for the first time. In 1962, Powell and Stallman found that detection of IgG I was associated with protracted convalescence from Q fever in the elderly. However, the authors stated that the presence of phase I antibodies was an indication of "past persistent infection" but that it was not necessarily proof of present persistent infection in the absence of clinical signs (382). One year later, the WHO study group on rickettsial diseases established a serological cutoff for phase I antibody titers at 1:200 for the diagnosis of "chronic Q fever" (383). Over the following years, both terms, "Q fever endocarditis" and "chronic Q fever," were used synonymously to describe one and the same entity (383–385), as stressed by an editorial in the Lancet in 1976 entitled "Chronic Q fever or Q fever endocarditis?" (386). It was previously observed that these cases of endocarditis affected mainly the aortic valve (387) and had a very poor prognosis, with frequent relapses after surgical valve replacement and treatment with tetracyclines alone (388). In 1978, Turck discussed the accuracy of the serological criteria for Q fever endocarditis developed by the WHO study group (389). This author pointed out that some cases of proven Q fever endocarditis (i.e., with the organism being isolated from valves postmortem) were reported with only slightly elevated levels of phase I antibodies (389). Also in 1978, Spicer suggested that the significance of phase I antibodies should be interpreted carefully, in conjunction with the medical history (383). In 1983, Ellis et al. reported a series of 16 cases of "chronic" Q fever, among which eight were not endocarditis but one of the following conditions: aortic prosthetic graft infections, spondylodiscitis with psoas abscess, infection of a ventricular aneurysm, infection during pregnancy, and unexpected death of a 9-month-old infant (390). that paper suggested early that *C. burnetii* infectious foci could be very diverse and that a wide range of clinical settings were artificially grouped under the generic term "chronic Q fever."

However, some years later, Peacock et al. reinforced the idea that IgG I serological cutoffs could differentiate between two restricted patterns of the disease: "primary Q fever" and "chronic Q fever," which could manifest as granulomatous hepatitis or endocarditis (391). That study included 15 patients (5 with primary infection, 5 with endocarditis, and 5 with "granulomatous hepatitis") and found that granulomatous hepatitis was associated with persistent elevated phase II IgG and that endocarditis was associated with both phase I and II elevated antibodies and high levels of IgA (391). A AQ: P second work by the same group with a larger sample of patients confirmed the association of phase I IgG with endocarditis (392). However, in this seminal study, the

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entity of "chronic granulomatous hepatitis" was not clearly defined. Actually, granulomas were more frequently described in previous reports in acute Q fever hepatitis, while chronic liver abnormalities were described mainly in the context of endocarditis. Moreover, early serological studies in countries where the disease was endemic failed to demonstrate that Q fever was a cause of chronic liver disease (383, 390). Until now, this entity of isolated "chronic granulomatous Q fever hepatitis" has been extremely rarely reported, and most of the reports employing this term actually deal with either acute granulomatous hepatitis or chronic hepatitis associated with endocarditis (372, 393–400). This early example illustrates that cautious definitions are mandatory in order to avoid considering artificial clinical entities.

Nevertheless, the accumulation of restricted serological criteria coupled with the lack of other powerful microbiological diagnostic tools induced a dissociation between "serological chronic Q fever" and possible clinical manifestations of the infection. This is illustrated by a report in 1985 dealing with "subclinical chronic Q fever" by Fergusson et al. In that paper, the authors described seven patients with high IgG I levels but without a detectable localized infection. In fact, the serological definition of "chronic Q fever" prevented the authors from diagnosing what can easily be considered today, with current classifications, as four possible cases of endocarditis, two possible infections of cardiac ventricular aneurysms, and one possible abdominal aortic aneurysm infection (401). Unfortunately, the damage from this misleading term has continued until very recently, discouraging clinicians from investigating *C. burnetii* infectious foci. In a case report from 2006, de Silva et al. (688) described two patients with "atypical chronic Q fever" considered to be without a focus of infection, which using current diagnostic criteria could be considered possible endocarditis and/or osteoarticular infection and thus should have led to more thorough investigations.

AQ: Q

Our team, like all specialists working on Q fever worldwide, also used the consensual "chronic Q fever" term in the past. In particular, we confirmed early on that endocarditis was associated with high levels of IgG1 antibodies (402), with titers of $>$800, and defined by a spontaneous progression longer than 6 months (337, 396). Also, we contributed to the modification of the Duke criteria, showing that the inclusion of this serological cutoff as a major criterion would improve the diagnosis of *C. burnetii* endocarditis (403, 404). Some years later we revaluated the PPV of this cutoff in the French situation of endemicity and proposed a new serological IgG1 cutoff at 1,600 to allow better detection of these cases of endocarditis (340). We also contributed to the early description of *C. burnetii* vascular graft, joint, and bone infections (394, 405), which were all grouped at that time under the generic "chronic Q fever" term. However, the reanalysis of the literature by a different team, leading to a corpus of controversial hypotheses, has helped us to clarify the existing nosology of *C. burnetii* infection and to abandon the inaccurate "chronic Q fever" term.

The controversy raised by Dutch Q fever consensus group guidelines. The recent widespread outbreak of Q fever in the Netherlands allowed us to reexamine the existing nosology of the infection, leading to continuing controversy on this topic. The total number of reported human cases of primary Q fever during this epidemic reached 4,108 between 2007 and 2011 (406). Moreover, because *C. burnetii* primary infection is frequently asymptomatic, these notifications reflected only part of the real magnitude of the outbreak (407). A seroprevalence study in blood donors by Van der Hoek et al. in a high-prevalence area estimated that 3,522 reported infections between 2007 and 2009 corresponded to approximately 44,000 primary *C. burnetii* infections (408). Consequently, the early detection of long-term complications of the infection was soon considered a central public health issue. With this in mind, in 2012 the Dutch consensus Q fever group published new criteria for "chronic Q fever" (409). The new guidelines distinguished three categories of possible, probable and proven cases of chronic Q fever (Table 2). Possible chronic Q fever was defined by an isolated serological criterion (IgG I greater than 1:1,024), and proven chronic Q fever was defined by a positive PCR on blood or tissue or by serological criteria associated with evidence of endocarditis (according to the modified Duke criteria) or vascular infections. Probable chronic Q

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TABLE 2 The Dutch consensus guidelines criteria for chronic Q fever[a]

Category and criterion (criteria)[b]
Proven chronic Q fever
Positive *C. burnetii* PCR in blood or tissue **or**
IFA titer of ≥1:1,024 for *C. burnetii* phase I IgG **and**
Definite endocarditis according to the modified Duke criteria **or**
Proven large-vessel or prosthetic infection by imaging studies (18 F-FDG PET, CT, MRI, or AUS)
Probable chronic Q fever
IFA titer of ≥1:1,024 for *C. burnetii* phase I IgG **and** one or more of the following criteria:
Valvulopathy not meeting the major criteria of the modified Duke criteria
Known aneurysm and/or vascular or cardiac valve prosthesis without signs of infection by means of TEE/TTE, 18 F-FDG PET, CT, MRI, or abdominal Doppler ultrasound
Suspected osteomyelitis or hepatitis as manifestation of chronic Q fever
Pregnancy
Symptoms and signs of chronic infection such as fever, wt loss, and night sweats, hepatosplenomegaly, persistent elevated ESR and CRP
Granulomatous tissue inflammation, proven by histological examination
Immunocompromised state
Possible chronic Q fever
IFA titer of ≥1:1,024 for *C. burnetii* phase I IgG without manifestations meeting the criteria for proven or probable chronic Q fever

[a]Adapted from reference 409 with permission of Elsevier.
[b]MRI, magnetic resonance imaging; AUS, abdominal ultrasonography; ESR, erythrocyte sedimentation rate.

fever consisted of the association of serological criteria with a myriad of clinical situations from pregnancy to the immunocompromised state or evidence of granulomatous lesions (Table 2). Actually, several important issues were raised by these new criteria and were pointed out by our team (410).

(i) Isolated serological criteria for diagnosis are simplistic. In the Dutch consensus guidelines, the "possible chronic Q fever" category is based solely on a serological criterion without clinical manifestation or notion of follow-up duration. The problem with such a definition is that IgG I titers can vary widely, depending on the clinical situation and *C. burnetii* strain involved (238). For example, the MST 17 *C. burnetii* strain from Cayenne, French Guiana, is responsible for high levels of phase I IgG in acute Q fever pneumonia patients. We showed in a previous work that 36% of patients from Cayenne, French Guiana, with acute Q fever had phase I IgG titers of >1:1,600 (238), so these patients could have been falsely classified as having "possible chronic Q fever" with the Dutch definition. The other risk of serological cutoffs when they are considered alone is that cases of "possible chronic Q fever" are not thoroughly investigated to find a focus of infection, because the recommended management in this situation is only "follow-up." On the contrary, investigations are necessary to identify a nosologic entity that allows determination of the most appropriate treatment (including the need for dual antibiotherapy or surgery) and its duration. Moreover, today new diagnostic tools such as 18-fluoro-2-deoxyglucose positron emission tomography/computed tomography (18 F-FDG PET/CT) are available and frequently allow localization of the infection (411, 412). Thus, we consider that this "possible chronic Q fever" category is not only useless but deleterious, because classifying a patient under this imprecise term can lead to neglecting a real persistent focus of infection.

AQ: S

(ii) Mixing different clinical entities under a generic term neglects the natural history and determinants of the disease. The term "chronic Q fever" is misleading, because it mixes very different clinical entities that warrant different strategies for prophylaxis, diagnosis, and treatment. this gave us the opportunity to reanalyze how we consider the natural history of *C. burnetii* infection (Fig. 4). We think that this natural history is quite similar to what happens in tuberculosis (TB). In TB, the primary infection can be symptomatic or not. In the absence of treatment and depending on host susceptibilities, persistent infection can develop, with various possible localizations (lymphadenitis, miliary, meningitis, or Pott's disease) that have to be detected in order

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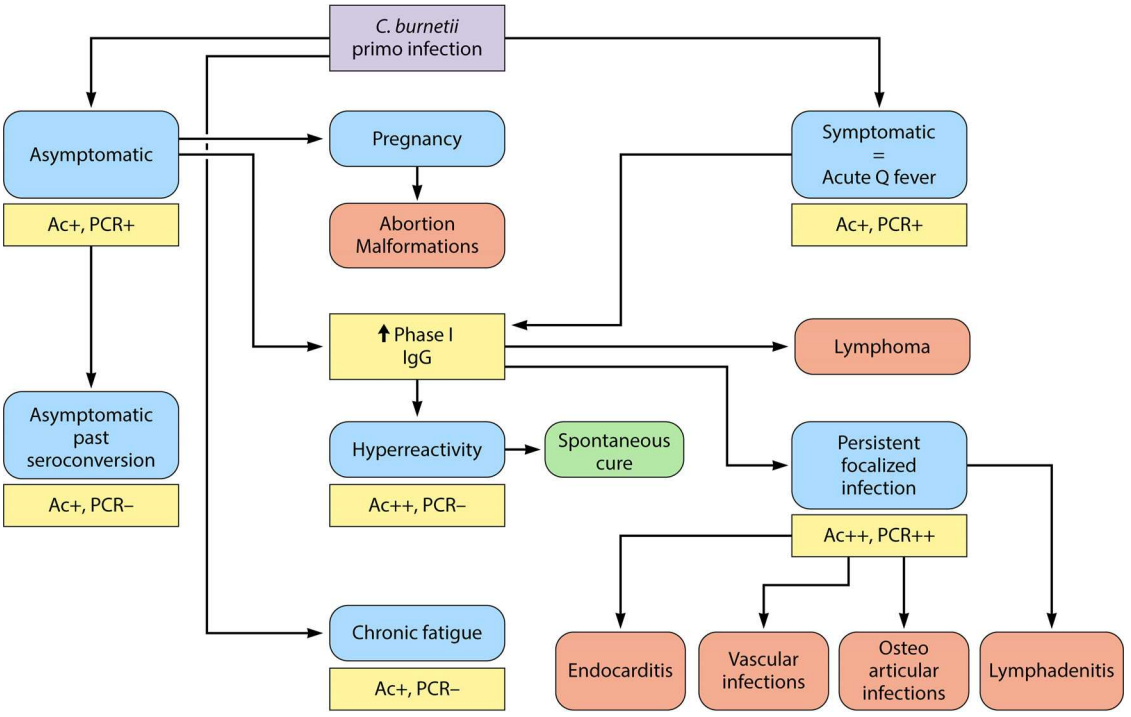

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FIG 4 Natural history of *C. burnetii* infection.

to determine a specific treatment strategy. Regarding *C. burnetii* infection, the primary infection can also be symptomatic (acute Q fever) or not, depending on the strain involved (144, 165) and on the patient's susceptibilities (due to age, sex, immunosuppression, or pregnancy) (413). If left untreated, diverse persistent focalized infections can develop, depending mainly on host susceptibilities.

C. burnetii endocarditis occurs in patients with preexisting valvulopathy (mainly bicuspid aortic valve) (414) and is initially associated with high levels of anticardiolipin antibody (320). Its prognosis has significantly improved, due to a strategy of systematic screening for valvulopathy in cases of primary *C. burnetii* infection and initiation of prophylaxis (415). Another argument against the acute/chronic dichotomy is the recent description of "acute" endocarditis caused by *C. burnetii* (416). This is a new clinical entity, due to a probable autoimmune mechanism during *C. burnetii* primary infection.

Vascular infections occur in patients with preexisting aneurysms of a vascular graft and remain very severe diseases (with mortality rates up to 25%), for which surgery appears to be mandatory (417, 418). In this group, *C. burnetii* infections of preexisting aneurysms or vascular grafts have to be distinguished from mycotic aneurysms resulting from septic emboli in *C. burnetii* endocarditis. Osteoarticular infections are an emerging clinical entity for which no deaths have been reported to date (419). Joint prosthesis may be a predisposing factor, but further studies are needed to confirm this (420). Persistent lymphadenitis is another recognized focus of infection (412).

Also, we recently showed that *C. burnetii* persistent infections could lead to lymphomagenesis (334). *C. burnetii* infection in pregnant women is a particular entity, most often with an asymptomatic primary infection that can lead to severe obstetrical complications and fetal malformations (148) (Fig. 4). Its outcome may also depend on the involved strain (164).

Given the polymorphic manifestations of *C. burnetii* infection, we proposed alternative definitions for all *C. burnetii* persistent infectious foci (Table 3) (410, 412, 420). AQ:T/T3

For each localization of persistent infection, we defined minor, major, and definite criteria, and association with these criteria leads to definite or possible diagnosis. Definite criteria for Q fever include isolation of *C. burnetii* in culture or detection by

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TABLE 3 Definition criteria for *C. burnetii* persistent focalized infections

Criterion or diagnosis type	Definition for *C. burnetii*: Endocarditis	Vascular infection	Prosthetic joint arthritis	Osteoarticular infection (without prosthesis)	Lymphadenitis
Criteria					
Definite	Positive culture, PCR, or immunochemistry of a cardiac valve	Positive culture, PCR, or immunochemistry of an arterial sample (prosthesis or aneurysm) or a periarterial abscess or a spondylodiscitis linked to aorta	Positive culture, PCR, or immunochemistry of a periprosthetic biopsy specimen or joint aspirate	Positive culture, PCR, or immunochemistry of bone or synovial biopsy specimen or joint aspirate	Positive culture, PCR, immunohistochemistry, or fluorescence *in situ* hybridization of lymphadenitis
Major	Microbiology—positive culture or PCR of the blood, an embolus or serology with IgG1 antibody titer of ≥6,400 mg/dl. Evidence of endocardial involvement—(i) Echocardiogram positive for infective endocarditis: oscillating intracardiac mass on valve or supporting structures, in the path of regurgitant jets, or on implanted material in the absence of an alternative anatomic explanation; abscess; new partial dehiscence of a prosthetic valve; or new valvular regurgitation (worsening or changing of preexisting murmur is not sufficient). (ii) PET scan displaying a specific valve fixation and mycotic aneurism.	Microbiology—positive culture, PCR of the blood or emboli, or serology with IgG1 antibody titer of ≥6,400 mg/dl. Evidence of vascular involvement—(i) CT scan: aneurysm or vascular prosthesis + periarterial abscess, fistula, or spondylodiscitis. (ii) PET scan specific fixation on an aneurism or vascular prosthesis.	Microbiology—(i) Positive culture or PCR of the blood. (ii) Positive *Coxiella burnetii* serology with IgG1 antibody titer of ≥6,400 mg/dl. Evidence of prosthetic involvement—(i) CT scan or MRI positive for prosthetic infection: collection or pseudotumor of the prosthesis. (ii) PET scan or indium leukocyte scan showing a specific prosthetic hypermetabolism consistent with infection.	Microbiology—(i) Positive culture or positive PCR of the blood. (ii) Positive serology with IgG1 antibody titer of ≥800 mg/dl. Evidence of bone or joint involvement—(i) Clinical arthritis, osteitis, or tenosynovitis. (ii) CT scan or ultrasonography (for joint) or MRI: osteo-articular destruction, joint effusion, intra-articular collection, spondylodiscitis, synovitis, acromio-clavicular localization. (iii) PET scan or indium leukocyte scan showing a specific osteo-articular uptake.	Microbiology—(i) Positive culture or positive PCR of the blood. (ii) Positive serology with IgG antibody titer of ≥800 mg/dl. Evidence of lymph node involvement—(i) Clinical lymphadenitis. (ii) CT scan or ultrasonography (for joint) or MRI: lymphadenitis of >1 cm. (iii) PET scan showing specific lymph node uptake.
Minor	Predisposing heart condition (known or found on ultrasound). Fever, temp of >38°C. Vascular phenomena, major arterial emboli, septic pulmonary infarcts, mycotic aneurysm (observed during PET scan), intracranial hemorrhage, conjunctival hemorrhages, and Janeway lesions. Immunologic phenomena: glomerulonephritis, Osler's nodes, Roth spots, or rheumatoid factor. Serological evidence: IgG1 antibody titer of ≥800 and <6,400 mg/dl.	Serological IgG1 antibody titer of ≥800 and <6,400 mg/dl. Fever, temp of ≥38°C. Emboli. Underlying vascular predisposition (aneurysm or vascular prosthesis).	Presence of a joint prosthesis (indispensable criterion). Fever, temp of >38°C. Joint pain. Serological evidence: positive *C. burnetii* serology with IgG1 antibody titer of >800 and <6,400 mg/dl.	Serological IgG1 antibody titer of ≥400 and <800 mg/dl. Fever, temp of ≥38°C. Mono- or polyarthralgia.	Serological IgG1 antibody titer of ≥400 and <800mg/dl. Fever, temp of ≥38°C.
Diagnoses					
Definite	(i) 1 definite criterion, (ii) 2 major criteria, or (iii) 1 major criterion and 3 minor criteria (including 1 microbiological characteristic and a cardiac predisposition)	(i) 1 definite criterion, (ii) 2 major criteria, or (iii) 1 major criterion and 2 minor criteria (including 1 microbiological characteristic and a vascular predisposition)	(i) 1 definite criterion, (ii) 2 major criteria, or (iii) 1 major criterion and 3 minor criteria (including 1 piece of microbiology evidence and presence of a joint prosthesis)	(i) 1 definite criterion, (ii) 2 major criteria, or (iii) 1 major criterion and 3 minor criteria (including 1 microbiological characteristic)	(i) 1 definite criterion, (ii) 2 major criteria, or (iii) 1 major criterion and 2 minor criteria (including 1 microbiological characteristic)
Possible	(i) 1 major criterion and 2 minor criteria (including 1 microbiological characteristic and a cardiac predisposition) or (ii) 3 minor criteria (including 1 microbiological characteristic and a cardiac predisposition)	Vascular predisposition, serological evidence, and fever or emboli	(i) 1 major criterion and 2 minor criteria (including 1 piece of microbiology evidence and presence of a joint prosthesis) or (ii) 3 minor criteria (including positive serology and presence of a joint prosthesis)	(i) 1 major criterion and 2 minor criteria or (ii) 3 minor criteria	(i) 1 major criterion and minor criterion or (ii) 2 minor criteria

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molecular biology or immunochemistry of *C. burnetii* in the affected organs (Table 3). Major criteria include indirect microbiological evidence of infection (positive serology or positive PCR on blood) associated with compatible morphological abnormalities, with 18 F-FDG PET/CT playing a central role in this setting. Minor criteria associate serological evidence, unspecific clinical signs of infection, and predisposition to the suspected focus of infection (e.g., known vascular aneurysm or graft in the case of *C. burnetii* vascular infection).

Apart from this corpus of persistent focalized infections with evidence of multiplying *C. burnetii*, a long-term complication of the infection can manifest as "chronic fatigue syndrome" (CFS) (Fig. 4).

(iii) Prevention strategies and prognosis for *C. burnetii* infection depend on the definition and understanding of the natural history of the disease. In a recent publication, the Dutch Q fever consensus group argued against the definition of persistent *C. burnetii* infections proposed by our team (endocarditis and vascular infections) by comparing definition criteria applied to the patients from the Dutch National Chronic Q fever database (421). They observed that some patients diagnosed with "proven chronic Q fever" in their cohort would have been missed by the criteria proposed by our team. Particularly, six patients from their cohort (4 with endocarditis and 2 with vascular infections) died of "chronic Q fever" and would not have been diagnosed by our alternative criteria. First, this study suffers from a bias, which is the use of our criteria *a posteriori*, because the definition of patients had already been made from their criteria. Consequently, our criteria could only show lower performances. Moreover, different definitions lead to different management of the infections. In the cases they presented, even if patients had been diagnosed with "chronic Q fever" from their criteria, they died of *C. burnetii* endocarditis and vascular infections. Indeed, regarding *C. burnetii* endocarditis, our strategy of systematic echocardiography to detect significant silent valvulopathy and initiating 12 months of prophylactic treatment with doxycycline plus hydroxychloroquine has proven effective (320). In 31 patients followed in our center who were diagnosed with significant valvulopathy during primary infection, 18 patients completed a 12-month course of antibiotic prophylaxis and 13 patients did not. We observed no endocarditis in the group with antibiotic prophylaxis, while all 13 patients who did not receive antibiotic prophylaxis progressed to endocarditis (320). The efficacy of such antibiotic prophylaxis is also illustrated by the fact that we observed a reduction in the incidence of *C. burnetii* endocarditis in our center over a 27-year period, despite a concomitant increase in the number of diagnosed primary infections (422). We believe that our management strategy would have allowed a lower mortality rate in this cohort of patients, because death due to endocarditis would have been prevented.

Conversely, in the Netherlands, echocardiographic screening of patients with primary *C. burnetii* infection was abandoned early, due to a 1-year follow-up study by Limonard et al. showing no cases of endocarditis despite detection of valvulopathy in 59% of patients (423). One of the arguments advanced was the unfavorable cost-effectiveness ratio of echocardiography in the context of their epidemic. We consider that such a decision was very premature, given the potential long-term consequences of *C. burnetii* endocarditis for public health. In another Dutch study, 62% of patients classified as having "proven" or "probable" chronic Q fever had a symptomatic primary infection, with 28% of them having a predisposing valvulopathy and 62% of them having a minor echocardiographic criterion (424). Unfortunately, none of these patients benefited from antibiotic prophylaxis because they were not screened during the primary infection. Recently, Keijmel et al. performed a retrospective case-control study on the diagnosis of acute Q fever in the Netherlands. That study found that 50% of patients with acute Q fever and risk factors (significant valvulopathy) developed endocarditis if no prophylaxis was given (425). This is a worrisome result, since a large number of patients with indication for prophylaxis may have been missed, due to insufficient screening of valvulopathy from the beginning of the epidemic.

We recommend close serological follow-up of all patients with *C. burnetii* primary

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infection at 3 and 6 months for early detection of an increase in antibody titers and patient investigation using 18 F-FDG PET-CT. This strategy has been abandoned in some centers in the Netherlands, with a serological control performed only 9 months after primary Q fever (426).

The same controversy exists about the management of *C. burnetii* infection during pregnancy. We previously described that infection during pregnancy could lead to severe obstetrical and neonatal complications (427, 428). We recently performed a meta-analysis confirming that these infections, although frequently asymptomatic, were associated with miscarriage, fetal death, malformations, and prematurity (148). To avoid these severe complications, we recommend treatment with co-trimoxazole until the eighth month of pregnancy (164). A recent Danish study confirmed such associations, with 47% of pregnant women seropositive for *C. burnetii* presenting obstetrical complications (miscarriage, preterm delivery, small infant for gestational age, oligohydramnios, fetal growth restriction, or perinatal death) (313). Interestingly, previous large case-control studies in the same country using serological markers of past *C. burnetii* infection in pregnant women failed to detect an association with obstetrical complications (429, 430). In the Netherlands, a randomized controlled study of serological screening in pregnant women in high-prevalence areas was performed in 2010 and found no advantage of screening in terms of prevention of obstetrical complications (431). As a consequence, systematic screening for *C. burnetii* infection in pregnant women in high-risk areas was abandoned in the Netherlands. As in the case of endocarditis, this decision seems quite premature, given the available data from the literature. Also, a comment from E. Leshem in 2012 about this strategy noted that this decision could expose unprotected medical personnel (especially obstetrical staff) to infection by inhalation of high concentrations of *C. burnetii* from the placentas of infected women (432). To date, the consequences of such a strategy are unknown.

Finally, regarding the particular severity of *C. burnetii* vascular infections, we recently proposed systematic screening for vascular aneurysm in men older than 65 years presenting with primary Q fever. We proposed that a CT scan or abdominal ultrasound (in case of renal contraindication) be performed for these patients in order to initiate prophylactic treatment, similar to what has been done for endocarditis (418).

In conclusion, this controversy illustrates the importance of nosology in the management of infectious diseases. In the particular setting of *C. burnetii* infection, which has long been considered a rare and mysterious disease, it is necessary to consider updated data resulting from clinical observations in the field. Definitions must play the role of practical tools, assisting clinicians' decisions, and must not consist of misleading terms lacking a microbiological substratum. In the following section we provide a detailed description of each clinical entity caused by *C. burnetii* infection.

The other current definition criteria for "chronic Q fever." As a consequence of this controversy, several different definitions for "chronic" or "persistent focalized" infection can be found in the recent literature. In addition to the definition from the Dutch consensus guidelines and the one proposed by our team, there is a third definition, which was proposed by the CDC in 2013 (Table 4). This definition uses the terms "probable Q fever" and "confirmed chronic Q fever." "Probable Q fever" is defined by the existence of a clinical presentation compatible with this diagnosis, such as culture-negative endocarditis or vascular aneurysm or graft infection (Table 4), combined with an IgG I titer of $<$800. "Confirmed Q fever" is defined by the same clinical picture combined with laboratory confirmation with serological titers of $>$800 or detection of *C. burnetii* by culture, PCR, or IHC in a clinical sample. Compared to the Dutch definition, it has the advantage of taking into account the suspected localization of the infection as an important criterion. However, it continues to use the term "chronic Q fever," which we consider to be confusing, now that *C. burnetii* foci of infection are well described.

AQ:U/T4

Clinical Manifestations

Primary infection. *C. burnetii* primary infection can manifest itself through a wide diversity of clinical symptoms. The incubation period for the primary infection before

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TABLE 4 Definition criteria for "chronic Q fever" from the CDC[a]

Category	Description
Indications	Newly recognized culture-negative endocarditis (particularly in a patient with previous valvulopathy or compromised immune system), suspected infection of a vascular aneurysm or vascular prosthesis, or chronic hepatitis, osteomyelitis, osteoarthritis, or pneumonitis in the absence of other known etiology
Laboratory confirmed	One or more of the following criteria: IgG titer of ≥1:800 to *C. burnetii* phase I antigen by IFA, detection of *C. burnetii* DNA in a clinical specimen by PCR, demonstration of *C. burnetii* in a clinical specimen by IHC, isolation of *C. burnetii* from a clinical specimen by culture
Laboratory supportive	IFA IgG titer of ≥1:128 and <1:800 to *C. burnetii* phase I antigen
Confirmed chronic Q fever	Clinical evidence of infection with laboratory confirmation
Probable chronic Q fever	Clinical evidence of infection with laboratory supportive results

[a]Adapted from reference 40.

the onset of symptoms can last from 2 to 3 weeks, and it depends on the size of the inoculum. In a large proportion of patients, the primary infection can be asymptomatic (433). In other cases, pneumonia, hepatitis, or flu-like syndrome can be observed.

However, these extremely polymorphic features of *C. burnetii* primary infection are not predictive of the development of long-term complications, such as persistent focalized infection or chronic fatigue syndrome (Fig. 4).

This issue has been raised by a recent study in the Netherlands. The European Union case definition for reporting Q fever used during the outbreak prevented the reporting of asymptomatic cases, leading to an underestimation of the true burden of the disease (407, 434) and to a probable significant lack of prevention of long-term complications for at-risk patients.

The determinants of the symptomatology in *C. burnetii* primary infection depend on host factors and on the strain involved. Tissot-Dupont et al. long ago showed the role of age and sex in the clinical expression, with older men being more frequently symptomatic than young women and pregnant women (413, 435). This was recently illustrated during the Netherlands epidemic, with symptomatic patients being significantly older and more often men than asymptomatic patients (407). Children are also less frequently symptomatic than adults (310). The role of immunosuppression in the severity of *C. burnetii* primary infection is not well defined. An old report of an outbreak in a residential facility for drug users in Italy suggested that respiratory signs were more frequent in HIV-positive patients and that the incidence of infection was significantly higher in this population (436). A study in Marseilles also suggested a more frequently symptomatic primary infection in HIV patients than in the general population (437). However, other studies have shown a similar seroprevalence of primary *C. burnetii* infection in HIV and non-HIV patients, with no significant difference in terms of symptomatology (438–440). In Cayenne, French Guiana, patients with *C. burnetii* community-acquired pneumonia were not more frequently diabetic, HIV positive, or on corticosteroid treatment than patients with community-acquired pneumonia of other etiologies (80). A recent study among patients with rheumatoid arthritis in the Netherlands found no difference in seroprevalence or symptomatology in patients on anti-TNF-α therapy (441).

The strain of *C. burnetii* involved is the second determinant of the clinical manifestations of primary infection. This phenomenon can be illustrated by the example of the MST 17 clone, the unique genotype responsible for *C. burnetii* infection in Cayenne, French Guiana. This clone causes higher rates of symptomatic cases than clones from metropolitan France. In a recent outbreak in a military camp, it was shown that 100% of contaminated patients were symptomatic, illustrating the particular virulence of this genotype (unpublished data).

(i) Asymptomatic and pauci-symptomatic primary infection. Classically, it is stated that approximately 60% of individuals are asymptomatic during *C. burnetii* primary

infection (433). Mild symptomatic forms resemble a common cold and are often diagnosed retrospectively or during outbreaks through systematic testing. This phenomenon was soon illustrated by the discrepancy between seroprevalence studies and rates of symptomatic cases of Q fever during early outbreaks in Montana and Switzerland (442, 443). A more recent study performed in Denmark showed a rate of 64% of patients with asymptomatic primary infection (444). In the Netherlands, Hogema et al. found a rate of 12.2% of blood donors positive for anti-*Coxiella* IgG, resulting in an incidence rate of seroconversion of 5.7% per year, which is more than 10 times higher than the local number of symptomatic diagnosed cases per year in this region (445). This issue is of particular concern in these settings of epidemicity, since it has been demonstrated that *C. burnetii* can survive and remain infectious for several weeks in stored human blood samples (132).

(ii) Acute Q fever: isolated febrile syndrome or flu-like illness. In acute Q fever with isolated febrile syndrome or flu-like illness, the abrupt onset of high fever (often up to 40°C) is the predominant sign, which can last for more than 15 days and is frequently associated with myalgia and headache, mostly retro-orbital (433, 446). It was the most frequent clinical presentation in reported acute Q fever patients during the outbreak in the Netherlands (423). This nonspecific presentation is misleading and supports the fact that clinicians should include screening for *C. burnetii* primary infection in the presence of an isolated fever of unknown origin. Specific examination is crucial in this context, searching for epidemiological risk factors (rural setting, occupation, and contact with ruminants or parturient mammals).

(iii) Acute Q fever: pneumonia. The prevalence of pneumonia during primary infection is highly variable. In Cayenne, French Guiana, *C. burnetii* MST 17 is responsible for the highest rate of community-acquired pneumonia in the world (around 40%) (238). It is also the predominant presentation in the Maritime provinces of Canada (447), northern Spain (448), Croatia (449), and the Netherlands (72). In Cameroon, *C. burnetii* was responsible for up to 10% of community-acquired pneumonia (60, 450, 451). During the Netherlands epidemic, pneumonia was present in up to 86% of hospitalized patients with acute Q fever (452).

Q fever pneumonia usually presents in middle-aged men, often with no comorbid conditions (446). Acute pneumonia typically combines fever, cough, dyspnea, and auscultation abnormalities. It is frequently associated with extrapulmonary signs, such as myalgia, arthralgia, relative bradycardia, sore throat, chills, vomiting, abdominal pain, nausea, diarrhea, or constipation (446, 453, 454). Patients are also more likely to complain of headache (80, 452), with some reports mentioning the headache as "the most severe pain they ever had" (446). This sign has been reported in up to 40.5% of patients with *C. burnetii* primary infection (455). Less frequently, a skin rash or neurological signs can be observed (confusion, prostration, or Guillain-Barré syndrome) (446, 452).

Regarding laboratory findings, leukocyte counts are generally normal or low compared with those in pneumonia caused by other microorganisms (80, 446, 452, 456). Conversely, C-reactive protein (CRP) levels are typically high (452) and significantly higher than in other pneumonias (80, 425). In a report from the Netherlands, elevated liver enzymes were reported in 32.3% of patients (452), and this was also reported in 60% of patients with acute Q fever in Croatia (449).

Radiological findings are highly polymorphic. Several reports found rounded opacities (446, 457, 458) with a halo sign suggestive of an angio-invasive process, but classical interstitial or segmental opacities involving the lower lobes are also common (459). CT scanning has shown lobar, segmental, multilobar, or patchy involvement, sometimes associated with lymph node enlargement (446). More severe forms, such as necrotizing pneumonia, have been described in the immunocompromised patient (458). Pleural effusions are also described (80, 458) and have been reported in 9.9% of patients with *C. burnetii* pneumonia from Europe and North America (460). Exceptional cases of lung pseudotumor have been described (394, 461, 462), with a good response to doxycycline.

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The prognosis of *C. burnetii* pneumonia is usually favorable, with resolution of symptoms within 30 days (446). In the Netherlands, patients hospitalized for *C. burnetii* pneumonia had significantly lower CURB-65 scores than patients with other community-acquired pneumonias (452). However, respiratory distress syndrome leading to death can occur and has been described in Cayenne, French Guiana (80). The mortality rate reported in the Netherlands was approximately 1% in two studies (452, 463). Death was more frequent in patients with comorbidities.

(iv) Acute Q fever: hepatitis. Isolated hepatitis is a frequent presentation of acute Q fever. It is more frequent than pneumonia in countries where the disease is endemic, such as France (238), Spain (464), Israel (453), Portugal (459), and Taiwan (454, 465). Elevated liver enzymes are associated in almost all cases with fever, chills, and headache (465). Headache was even proposed in early reports for differentiating *C. burnetii* hepatitis from viral hepatitis (466). Other accompanying findings are anorexia, vomiting, and sometimes diarrhea and painful hepatomegaly (238, 453, 459, 464). Jaundice is rare, but it has been reported in severe hepatitis, especially in Taiwan, where hyperbilirubinemia was found in more than one-third (37%) of cases (465). In that study, patients with jaundice presented a significant delay in reduction of fever after initiation of antibiotic therapy compared to patients without jaundice (11.5 versus 5.0 days; $P = 0.002$) (465). Globally, the mean duration of fever decrease after initiation of adequate antimicrobial therapy in Taiwanese patients was 10 days, and 8 patients experienced a fever lasting more than 28 days (465). This particular feature may explain the confusion in early reports between what could be considered acute or chronic Q fever hepatitis (467). In developing countries where coinfection with viral hepatitis is high, clinical manifestations of *C. burnetii* hepatitis do not seem to be more severe, but further studies are needed to evaluate this feature, since only one retrospective study in Taiwan has been performed on this presentation (468). When performed, positron emission tomography can reveal an intense diffuse uptake of the whole hepatic parenchyma (469, 470), helping to establish the diagnosis in the case of fever of unknown origin.

When liver biopsy is performed, granulomatous hepatitis and typical "doughnut" granulomas are found (86). However, atypical pathological aspects have been reported recently, such as like epithelioid granuloma with eosinophilic infiltration, extensive extravasated fibrin without ring granuloma and acute cholangitis without granuloma (365). The prognosis of *C. burnetii* acute hepatitis is good. Fatal cases due to hepatic insufficiency are very rare and have been reported in a child (471), or in patients with cancer or alcoholism (472, 473).

(v) Acute Q fever: cardiac involvement. *(a) Pericarditis.* Acute pericarditis accounted for approximately 1% of Q fever cases diagnosed in our center (474) in the 1980s. This presentation has been reported as mimicking a lupus-like syndrome (475). The diagnosis of *C. burnetii* pericarditis has been improved with the use of a systematic prescription kit in our center (476), revealing that Q fever was involved in 24% of 81 cases of pericarditis investigated over an 8-year period (477). That study suggested that *C. burnetii* pericarditis is not as rare as previously postulated. The severity of these cases of pericarditis is variable, with reported cases of life-threatening tamponade (478) and some constrictive cases (478, 479).

(b) Myocarditis. Acute myocarditis is a life-threatening and fortunately rare form of primary infection. A total of 23 cases of acute myocarditis are reported in the English literature, (459, 474, 480–484), of which one involved a child (485). It represents 0.5% to 1% of cases diagnosed in our laboratory (305, 474). Among these 23 cases, 7 deaths (30%) were reported, illustrating the poor prognosis of this focalized infection. Although it is a rare manifestation, acute Q fever should be considered in the diagnosis of acute myocarditis because appropriate treatment may significantly improve the prognosis of this potentially fatal localization.

(c) Acute endocarditis. C. burnetii endocarditis is still considered the archetype of "chronic" Q fever. However, we recently reported cases of "acute endocarditis" (416). We observed a transient 10-mm aortic vegetation in a patient with primary Q fever,

associated with high levels of IgG anticardiolipin (aCL) 1 week after fever onset. This patient had no past medical history of valvular heart disease (VHD). This case prompted us to investigate this clinical presentation in patients followed in our National Referral Center and to compare this syndrome with classical primary Q fever in patients with or without preexisting VHD but without vegetation. We found 9 patients presenting features of "acute endocarditis" (416). All vegetations were localized to the aortic valve. Patients presented various associated clinical features such as isolated fever, pneumonia, or hepatitis. Regarding the prognosis, one patient died after 15 months from mesenteric infarction (long after successful treatment of primary Q fever), six patients progressed to *C. burnetii* persistent endocarditis, and two patients never fulfilled the criteria for persistent endocarditis and were treated for 12 months. The comparison between "acute endocarditis" and the other categories of primary infections found that predictors for this condition were immunosuppression and very high levels of IgG aCL (>100 IgG phospholipid units [GPLU]). The pathophysiological scenario for this new entity would be that *C. burnetii* primary infection causes an explosive secretion of autoantibodies, including IgG aCL, causing autoimmune valvular lesions. Further studies are needed to confirm and further characterize the features of this new clinical entity.

AQ: V

(vi) Acute Q fever: neurological signs. Neurological involvement is rare and can be observed alone or combined with other organ involvement. A total of 14 references concerning this presentation are found in the English literature, consisting mainly of case reports or small series (304, 474, 486–497). Apart from headache, which is a common sign in acute Q fever, revealing a possible neurological tropism of the bacterium (455), more severe manifestations such as meningitis and meningoencephalitis have been reported. Among 1,383 Q fever infections diagnosed in our reference center between 1985 and 1998, 1% had meningoencephalitis and 0.7% had meningitis alone (474). In a Greek report of 121 patients with Q fever consisting mainly of acute pneumonia, 4.1% presented with confusion and 0.8% had meningitis (455). Meningoencephalitis appears to be the most frequent acute severe neurological complication and can sometimes be the only manifestation of the disease, followed by meningitis and peripheral myelitis (491). Cerebrospinal fluid (CSF) findings show lymphocytic meningitis. Postinfectious neurological signs probably related to immunological disorders have been described, such as peripheral sensory neuropathy or Guillain-Barré syndrome (488, 490), with a good response to steroid therapy. The use of fluoroquinolones, which have good cerebrospinal penetration, has been proposed for treatment of *C. burnetii* meningitis (495).

(vii) Acute Q fever: rare clinical manifestations. *(a) Dermatological signs.* Cutaneous involvement was present in 4% of patients diagnosed with acute Q fever in our laboratory between 1985 and 1998 (474), and recent acute Q fever series have reported a prevalence ranging from 1% in the Netherlands, (452) to 9% in Israel (449, 453, 454, 498). These manifestations consist mainly of maculopapular or vesicular exanthema and sometimes purpuric lesions (474). Cases of granulomatous panniculitis (499) and erythema nodosum have also been reported (366, 500, 501).

(b) Bone marrow involvement. Although rarely reported, bone marrow lesions were already described in very old reports. The pathological analysis of bone marrow biopsy specimens shows a typical "doughnut" or "fibrin ring" granuloma during *C. burnetii* primary infection (253, 367–369, 469, 502–508). Also, a single case of bone marrow necrosis following acute Q fever was reported in 1980 (509). Hemophagocytic syndrome can be a consequence of bone marrow involvement. This clinical presentation was reported for the first time in 1984 by Estrov et al. (510), and since then fewer than 10 cases have been reported (511–513), with the most recent involving an asplenic patient (514). 18 F-FDG PET/CT, which is less invasive than bone marrow biopsy, can detect bone marrow involvement. A case of diffuse bone marrow increased uptake in the context of primary *C. burnetii* infection has been reported recently (515), and we found 11 patients with bone marrow hypermetabolism in the context of acute Q fever (412.

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(c) Acute lymphadenitis. Thirteen cases of acute Q fever lymphadenitis have been described, involving cervical, axillary, mediastinal, abdominal, or inguinal lymph nodes and associated with fever, headache, pneumonia, or hepatitis (458, 516–521). Also, in a recent study concerning the clinical relevance of 18 F-FDG PET/CT in *C. burnetii* infection, we observed seven cases of lymphadenitis in the context of primary Q fever, associated with bone marrow, lung, or splenic hypermetabolism. One patient with acute lymphadenitis was subsequently diagnosed with lymphoma (334).

(d) Cholecystitis. Cases of acute acalculous cholecystitis have been reported (522–527). A total of nine cases are reported in the literature. Patients present with right upper quadrant pain and fever. Abdominal CT scanning shows a diffuse symmetrically thickened and hypodense gallbladder. The diagnosis was made by serology in all cases. Six of the patients underwent cholecystectomy, and the pathology review showed associated fibrin ring granulomas in the liver for three of them. In one case, *C. burnetii* was detected by qPCR in the gallbladder (524).

(e) Autoimmunity. Biological markers of autoimmunity are frequently present in acute Q fever. Anti-smooth muscle antibodies, antineutrophil cytoplasmic antibodies (ANCA), and antinuclear and antiphospholipid antibodies have been detected during acute Q fever (292, 475, 475, 528–531). Lefebvre et al. have reported a series of seven cases of *C. burnetii* infection that could have been confused at initial presentation with Goodpasture's syndrome, Crohn's disease, Still's disease, polymyalgia rheumatica, polyarteritis nodosa, essential type II cryoglobulinemia, and giant-cell arteritis (531). Other authors have reported two cases mimicking exacerbations of systemic lupus erythematosus (475, 532).

AQ: W

Cases with positive antiphospholipid antibodies complicated by thrombophlebitis have been reported. This suggests that most clinical features of the antiphospholipid syndrome can be found in acute Q fever with laboratory classification criteria for the antiphospholipid syndrome (lupus anticoagulant and anticardiolipin antibody of IgG and/or IgM isotype) (686, 687; M. Million, N. Bardin, S. Bessis, N. Nouiakh, C. Douliery, S. Edouard, E. Angelakis, K. Griffiths, A. Bosseray, O. Epaulard, S. Branger, D. Chaudier, K. Blanc-Laserre, N. Ferreira-Maldent, E. Demonchy, F. Roblot, J. Reynes, F. Djossou, C. Protopopescu, P. Carrieri, H. Lepidi, L. Camoin-Jau, J.-L. Mege, and D. Raoult, unpublished data). Also, in the primary *C. burnetii* infection, high levels of IgG aCL antibodies and VHD have been associated with progression to persistent endocarditis (295). Rare cases of Q fever associated with amyloidosis (533) or mixed cryoglobulinemia (531, 534) are reported in the literature. Two case of Jarisch-Herxheimer reactions following the treatment of a *C. burnetii* pneumonia or endocarditis have been reported (535, 536).

AQ: X

***C. burnetii* persistent focalized infections. (i) Endocarditis.** Q fever endocarditis is the most frequently reported form of persistent *C. burnetii* infection in the literature. It is the most frequent persistent form in France (340) and the second most frequent one in the Netherlands (78). In Brazil, *C. burnetii* was found to be the etiological agent in approximately 10% of blood culture-negative endocarditis cases (685). In Israel, 9.6% of patients undergoing valve replacement for endocarditis had a *C. burnetii* infection (537). *C. burnetii* endocarditis has also been reported in 8.3% of 60 patients diagnosed with bacterial endocarditis in Thailand (538). In Africa, it represents from 1% to 3% of infective endocarditis in cohort studies (450). However, its prevalence is probably underestimated in most developing countries, where microbiological tools for diagnosis are lacking.

Factors associated with progression to endocarditis after primary infection are male sex, age above 40 years, and, most importantly, underlying valvular heart disease, even if clinically silent at diagnosis (320). The incidence of endocarditis after acute Q fever in patients with valvulopathy has been estimated to be 39% (539). Having a valvular prosthesis (mechanical or bioprosthesis) represents the most important risk factor, as initially reported by Fenollar et al. (539). Also, even minor valvulopathies are at risk, with the highest risk for aortic bicuspidy, followed by mitral valve prolapse and minimal valvular leaks (414, 540). Given the fact that the prevalence of bicuspidy has been estimated to be 1% to 2% in the general population, screening for this valvulopathy is

of particular importance in patients with primary Q fever (541). Regarding more severe valvulopathy, aortic regurgitation is at higher risk than mitral regurgitation and aortic stenosis (320). Also, three cases of *C. burnetii* infection of cardiovascular implantable electronic devices and a single case of *C. burnetii* infection of an intracardiac thrombus mimicking atrial myxoma have been reported to date (412, 542, 543).

The clinical presentation of *C. burnetii* endocarditis is nonspecific, and patients can present symptoms such as isolated relapsing fever, chills, night sweats, weight loss, and hepatosplenomegaly (433). Sudden cardiac insufficiency, stroke, or other embolic signs are also presentations of the disease (415). Mycotic aneurysms resulting from endocarditis can occur and must be distinguished from isolated vascular infection of a preexisting aneurysm (418, 544). Laboratory signs can consist of a persistent inflammatory syndrome and hyperleukocytosis or, on the contrary, pancytopenia (539, 545). Cases of coinfection with *Enterococcus faecalis*, *Streptococcus mitis*, and *Streptococcus gallolyticus* have been reported (415, 546–548).

Million et al. have reported presentations with valvular vegetations in only 30% of cases and discovery or worsening of a valvular insufficiency in 75% of cases, illustrating the difficulty in the diagnosis of Q fever endocarditis (415). This is illustrated by a study in Marseilles, where systematic screening of 6,401 patients undergoing valve surgery yielded an unexpected diagnosis of *C. burnetii* endocarditis in 14 patients (0.2%) (549). Also, Tyler et al. recently reported a case of *C. burnetii* endocarditis incidentally discovered during routine valve replacement in a patient with unexplained pancytopenia and splenomegaly (545), and similar cases were reported in the Netherlands by Kampschreur et al. (550). In most patients, the infection can be latent for years, while *C. burnetii* progressively destroys heart valves, causing irreversible damage (549). Million et al. have shown that high levels of IgG aCL during acute Q fever were associated with the presence of a valvulopathy and a predictive biomarker of progression to persistent endocarditis (295). In particular, IgG aCL levels higher than 90 IU were strongly associated with endocarditis.

Serological IgG1 titers of $>$1,600 are associated with endocarditis (340), and this cutoff has been added in the modified Duke criteria for endocarditis. However, some cases of endocarditis with IgG1 titers of $<$800 have been described (341), so that this criterion alone is not sufficiently accurate for diagnosis. Specific qPCR on blood or serum is positive in about 30% of cases, and blood culture is positive in 14% (415). The analysis of resected valves by culture, qPCR, immunohistochemistry, and pathology shows variable performances (from 0% to 87% positivity), depending on the time the resections are performed in relation to treatment.

Pathological examination of cardiac valves may reveal fibrosis and calcification, slight inflammation and vascularization, and minimal or absent vegetation (328). These signs can be confused with noninfectious cardiac valve damage, so immunohistochemical analysis is helpful to confirm the diagnosis (328).

18 F-FDG PET/CT scanning has been used as a new tool for detecting infected valves in *C. burnetii* endocarditis, and it seems to be particularly useful when no vegetation is present and in cases of prosthetic valve infection. Barten et al. have reported four cases of *C. burnetii* endocarditis, all with hypermetabolism in a prosthetic valve (551). A case report from Australia describes the diagnosis and follow-up of *C. burnetii* endocarditis in a patient with aortic bicuspidy with an 18 F-FDG PET/CT-scan (552). Also, the diagnosis of *C. burnetii* infection of a Bentall graft or prosthetic mitral or aortic valve with 18 F-FDG PET/CT scanning has been reported (553, 554). In a recent retrospective study from our center of 99 patients with *C. burnetii* infection and a positive 18 F-FDG PET/CT scan, we observed 21 patients with hypermetabolism of a cardiac valve, with a majority of them involving a prosthetic valve (66%) (412).

Because there is no pathognomonic sign or accurate paraclinical tool for the diagnosis of *C. burnetii* endocarditis, new criteria have recently been proposed, inspired by the modified Duke criteria (Table 3), incorporating PCR and culture on blood samples, echocardiography, and 18 F-FDG PET/CT scanning as major diagnostic criteria (410).

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The prognosis of *C. burnetii* endocarditis has considerably improved because of earlier diagnosis and appropriate dual-antibiotic therapy. In the Netherlands, Kampschreur et al. found mortality rates of 9.3% for *C. burnetii* endocarditis, (78) with acute presentation (severe endocarditis) being associated with a poor outcome. In our center, we found mortality rates of about 5% at 3 years, and factors independently associated with death were age, stroke, and prosthetic valve at diagnosis (415). At 1 year of follow-up, the absence of a 4-fold decrease of IgG1 and IgGA and persistence of IgM were also associated with death (415). We recommend performing systematic echocardiography in patients diagnosed with primary Q fever to detect any predisposing valvular lesions and, if such lesions are detected, providing regular monitoring and prophylactic treatment with doxycycline and hydroxychloroquine (540). We have demonstrated retrospectively in our cohort from the National Referral Center that this strategy is effective in preventing progression to endocarditis in all patients with valvulopathy who underwent complete treatment (320). Conversely, patients diagnosed with primary Q fever and valvulopathy who did not follow this prophylaxis systematically progressed to endocarditis (320).

(ii) Vascular infections. An increasing number of reports of *C. burnetii* vascular infections have been published in the last decade, mainly due to the outbreak in the Netherlands (78, 371, 405, 417, 551, 555–575). At present, it is the most frequent form of persistent infection reported in the Netherlands (371, 405, 417, 418, 553, 557–577). In that country, a seroprevalence study among patients with abdominal aortic/iliac aneurysm detected *C. burnetii* antibodies in 16.7% of them, of which 30% had serological titers suggesting persistent vascular infection (562). In France, it is the second most prevalent site of persistent infection after endocarditis (417, 474). In our center, we observed an increase in the incidence of vascular infections in the last 5 years compared to the 22 preceding years (418). This may be due to several factors: systematic screening of patients with aneurysm and vascular graft for whom biological samples are available in our laboratory, elaboration of a diagnostic score (Table 2), and new diagnostic tools such as the 18 F-FDG PET/CT scan (412). No data exist regarding prevalence in other countries, because the literature consists mainly of case reports.

These infections develop after *C. burnetii* primary infection when a preexisting lesion is present on a vessel, such as an aneurysm or vascular graft. The most frequent localization is the abdominal and thoracic aortas (417). Because *C. burnetii* vascular infections are latent and initially present with unspecific symptoms (weight loss or unexplained fever in a patient with an aneurysm or vascular graft), in the majority of the reported cases the diagnosis was made when complications occurred. The main complications are aortoduodenal fistulas (555, 558, 568) leading to catastrophic hemorrhage, spondylodiscitis (417, 565, 575) often associated with psoas abscesses (418), graft or aneurysm rupture (417), (78) and embolic complications (78, 417, 557). A single case of coinfection with *Yersinia enterocolitica* has been reported (411). The overall prognosis is poor, with mortality rates between 18% and 26% (78, 417, 418). In a recent Dutch study, the presence of vascular Q fever infection was significantly associated with mortality (78) in a cohort of patients with "chronic" Q fever.

As is the case for endocarditis, the diagnosis of vascular infection is challenging. IgG1 titers of >6,400 were observed in 45% of patients from our center, and the median IgG1 level was 3,200 (418). Specific qPCR on blood samples was positive in only 14% of patients. Culture and qPCR on vascular samples were positive in 58% and 91% of cases, respectively, sometimes after several months of antibiotic treatment (418). Pathological analysis of the vascular wall can show necrotizing granulomas (564). 18 F-FDG PET/CT scans have also been used on this indication in a total of 36 patients (of which 19 patients had a vascular graft) (411, 551). This exam has the advantage of detecting other sites of infection that are frequently associated, such as spondylodiscitis and psoas abscess.

To improve the early diagnosis of these infections, proposed diagnostic criteria with PCR, culture, serology, CT scanning showing an aneurysm or vascular prosthesis, and 18

F-FDG PET/CT scanning showing aneurysm or graft hypermetabolism were included as major criteria (410) (Table 3).

(iii) Osteoarticular infections. Bone and joint *C. burnetii* infections were considered a rare entity, occurring in 2% of Q fever cases in initial series (474). However, they have been increasingly reported in the last decade. Osteomyelitis, often multifocal, seems to be more a frequent presentation in children, with a total of 11 cases reported in the literature (577–583). In adults, the clinical presentation is more variable. Isolated osteomyelitis seems to be much less frequent, with only 2 cases in the literature, one involving the cheek (584) (but this case would not fulfill current criteria for *C. burnetii* osteoarticular infection because of the absence of microbiological proof and low IgG1 titers) and one bilateral tibio-femoral osteomyelitis in a 49-year-old man with a doughnut granuloma and serological titers compatible with persistent infection (585). Six cases of isolated spondylodiscitis (not associated with endocarditis or vascular infection) have been reported (412, 474, 586, 587). A total of eight cases of culture-negative prosthetic joint arthritis have been reported, seven of them involving the hip and one involving the knee (412, 419, 420).

Other localizations include tenosynovitis of the wrist (2 cases) (586) and of the tibia (1 case) (412), subacromial bursitis (3 cases) (412, 419), and coxitis, sacroiliitis, arthritis of the ankle and shoulder (412, 419, 474). The use of an 18 F-FDG PET/CT scan has been reported for *C. burnetii* osteoarticular infections, and we have proposed diagnostic criteria for prosthetic joint arthritis and other osteoarticular infections (412, 419, 420) (Table 3).

(iv) Persistent lymphadenitis. A total of 18 cases of persistent focalized lymphadenitis have been reported (334, 412, 517). One case was associated with a diagnosis of lymphoma. Four cases of isolated persistent lymphadenitis were diagnosed with 18 F-FDG PET/CT scanning. Other cases were isolated (2 cases) or associated with endocarditis (1 case), vascular infections (4 cases), or osteoarticular infections (6 cases) (412). We recently developed a diagnostic score for *C. burnetii* persistent lymphadenitis (412) (Table 3).

C. burnetii Infection in Special Populations: Pregnant Women and Children

***C. burnetii* infection during pregnancy.** Primary infection in pregnant women is most often asymptomatic (413). However, poor obstetrical outcomes have been described, mainly when *C. burnetii* infection occurs during the first trimester (428, 588). Seroprevalence studies in pregnant women show very variable rates in areas of endemicity: 0.15% in southeastern France (589), 3.8% in Canada (590), and 4.6% in London, United Kingdom (591). In Denmark, a seroprevalence rate of up to 47% was reported in pregnant women who were occupationally exposed to livestock, versus 4.8% in unexposed women (592). During the outbreak in the Netherlands, seroprevalence rates between 3.4% (593) and 9% were reported in high-prevalence areas, with a statistically significant correlation with proximity to an infected dairy goat farm (594).

Various obstetrical complications have been reported in pregnant women infected with *C. burnetii*, such as miscarriage, fetal death, malformations (omphalocele, hypospadias, Potter syndrome, congenital hydronephrosis, and syndactyly), growth retardation, oligohydramnios, and premature delivery (148, 164, 428). A case of nosocomial transmission between 2 women in an obstetrical ward has been reported (131), probably due to dissemination of spores from birth products. Prevention of nosocomial transmission consists of precautions such as wearing masks and gloves during placental manipulation and isolation in a single room for infected pregnant women (428, 432). The mechanism of early abortion involves placental abruption due to placentitis (428, 595), and *C. burnetii* can be isolated from the infected placenta (164).

Obstetrical complications have been reported mainly in case series from France, Spain, Canada, and Australia (148, 590, 595–599), while recent large-scale population-based serological studies from Germany (600), Denmark (429, 430), and the Netherlands (431, 593) have shown no increased risk of adverse obstetrical outcomes in seropositive pregnant women (601). A recent meta-analysis from our team of 136 cases and 7

population-based studies confirmed some key points: seropositivity and untreated Q fever during pregnancy are associated with fetal death, and antibiotic treatment prevents this complication (148). The discrepancy in previous studies can be explained by several factors. First, Angelakis et al. found that *C. burnetii* strains harboring the QpDV plasmid were associated with an increased risk of abortion, suggesting that genotypes from different geographical areas could induce different rates of obstetrical complications (164). Second, contradictory results in studies from the Netherlands and Denmark may be due to bias in study design. For example, in Denmark, the same authors observed poor obstetrical outcomes in a case series (313, 602) and no increased risk of adverse pregnancy events in population-based serological studies (429, 430).

***C. burnetii* infection in children.** Specific studies of clinical manifestations of Q fever in children are few. Globally, children are more frequently asymptomatic than adults and tend to present less symptomatic forms of the primary infection. Seroprevalence studies in the general child populations from different geographical areas are available and show very variable rates of seropositivity (603). In Queensland, Australia (413), a seropositivity rate of 2.5% has been found in children <15 years (604). In West Africa, a recent study found a seroprevalence rate for Q fever of 8.3% among 796 children (605). An early Japanese study found 32.7% seropositivity among 55 school children with an influenza-like syndrome (606).

Regarding clinical manifestations, a recent study in the Netherlands of 49 children revealed that the top five clinical symptoms of primary infection were influenza-like syndrome, lower and upper respiratory tract infection, malaise, and digestive signs (gastroenteritis-like symptoms). The outcome was favorable in all cases. To date, no case of persistent *C. burnetii* infection has been reported in children in the Netherlands, but based on the prevalence of congenital heart disease, at least 13 children in the highest-prevalence area may be at risk of developing endocarditis (603). As is the case in adults, the clinical presentation of primary infection is not specific and can mimic other classical childhood infections, and testing for Q fever is rarely performed by pediatricians (607).

However, cases of hepatitis, meningoencephalitis, pericarditis, myocarditis, lymphadenitis, skin rash, rhabdomyolysis, and hemolytic-uremic syndrome in children caused by *C. burnetii* primary infection have been reported (304, 310, 608–614).

The most frequently reported persistent infection in children is osteomyelitis, which is frequently multifocal, with evidence of granulomatous bone lesions (577–583). Cases of endocarditis have been described mainly in children with congenital heart disease (187, 310, 615). One case of infection of a bovine jugular vein conduit graft (616) and one case of multiple recurrent abscesses (617) have also been described.

Other Related Clinical Syndromes

Ischemic stroke and atherosclerosis. Recently, a case-control study was conducted in Spain among patients aged >65 years presenting with ischemic stroke (618). Positive serology for persistent *C. burnetii* infection was found in 14.5% of cases and 6% of controls ($P < 0.004$). This association was conserved when adjusted for age, sex, and cardiovascular risk factors. Further studies are needed to investigate this association. The same authors found an association between past serological evidence of *C. burnetii* infection and cardiovascular atherosclerosis in the elderly (619). Before that, Lovey et al. had already found an association in a Swiss cohort between primary *C. burnetii* infection and the risk of developing a cerebrovascular accident and cardiac ischemia (620).

Lymphoma. Our team has recently demonstrated that *C. burnetii* infection is associated with an increased risk of lymphoma (334). Before that study, 22 cases of lymphoproliferative disease in the context of Q fever were available in the literature (621–624). A patient who was followed in our center for a *C. burnetii* vascular infection was diagnosed with B-cell lymphoma, and *C. burnetii* was detected in the lymphoma tissue. Therefore, we performed a retrospective study among all patients diagnosed in the French national reference center with Q fever to search for other cases of *C. burnetii*-associated lymphoma. A total of seven cases were found, presenting mature

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B-cell lymphoma (6 diffuse and one low-grade B-cell lymphoma). An excess risk of diffuse B-cell lymphoma and follicular lymphoma was found in Q fever patients compared to the general population (standardized incidence ratios [95% confidence intervals {CI}], 25.4 [11.4 to 56.4] and 6.7 [0.9 to 47.9], respectively). Moreover, patients with *C. burnetii* persistent focalized infections were more at risk of lymphadenitis and progression to lymphoma (odds ration [OR], 14.54; 95% CI, 2.14 to 337.7; $P = 0.007$). Consequently, *C. burnetii* may be a cofactor for lymphoma, and the possible mechanism could be the infection of monocytes and dendritic cells, causing impairment of the immune system leading to lymphoma.

CFS. Chronic fatigue syndrome (CFS) is usually described several years after primary infection with *C. burnetii*. The symptoms are characterized by persistent fatigue following a primary infection with no sign of persistent infection, and it has been described as Q fever fatigue syndrome (QFS). Nevertheless, cautious analysis of the literature is necessary regarding QFS, because the definition of this syndrome has been variable through the years. In particular, the first case-control studies from Australia and the UK showed a wide range of clinical signs, such as persistent fatigue, increased sweating, blurred vision, arthralgia, alcohol intolerance, breathlessness, or enlarged painful lymph nodes (625, 626). Moreover, no details are given about serological follow-up, so that symptoms attributed to QFS could in fact be related to undiagnosed persistent *C. burnetii* infection. This is illustrated by the study by Wildman et al. that took place 10 years after an outbreak in the UK. Among the 108 patients followed, some died of aneurysm rupture and endocarditis with cardiac failure, suggesting possible undiagnosed persistent infection, but no data were given about serological titers for these patients (627). In some studies, confusion between "chronic Q fever" and QFS is really tangible, because a proportion of the patients have a positive PCR for *C. burnetii* on blood samples or high IgA1 levels, so that no conclusion, in particular about the efficacy of antibiotics in this syndrome, can be drawn from them (628–631).

However, even if these studies suffer from bias, all have shown a significantly higher frequency of patients reporting fatigue after a primary *C. burnetii* infection than that found in healthy controls. Moreover, two studies have reported significant rates of *C. burnetii* seropositivity in cohorts of patients with CFS (27% and 17%) (632, 633). In these first studies, patients were tested with questionnaires inspired from the CFS criteria of the CDC (1994) (634). CFS was defined as a fatigue lasting for 6 months or more with elimination of somatic disease (hypothyroidism, narcolepsy, sleep apnea, drugs, chronic viral hepatitis, alcoholism, psychiatric trouble, or obesity) (634).

More recent studies have used a battery of different validated tests for evaluation of health status (SF-36, Chalder fatigue scale score, SF-12, MFI, SOMS, CDC-SI, Whiteley index, F-Sozu, and OQ-45) with duration of follow-up from 27 months to 6 years. They found a higher frequency (from 32% to 54%) of fatigue symptoms and impaired quality of life in patients who had experienced an episode of primary Q fever than in healthy controls (635–637). Strauss et al. also found that patients with fatigue symptoms following Q fever in Germany were more frequently subject to hypochondriacal worries and beliefs and somatization (637). However, Thomas et al. found no significant association between *C. burnetii* seropositivity and psychiatric morbidity in a retrospective cohort of farmers from the UK (638).

Regarding the pathophysiological explanation for this syndrome, Helbig et al. found a significantly increased frequency of HLA-DRB1*11 in patients with QFS, suggesting an immunological mechanism, depending on the host susceptibility (639, 640). Similarly, Pentilla et al. found that peripheral blood mononuclear cells (PBMCs) from patients with CFS exhibited higher levels of IL-6 secretion than those from controls when stimulated with *C. burnetii* antigens (285). Other studies failed to identify specific patterns of immune gene expression in QFS (641, 642). Some studies from the UK have tried to identify antigenic residual particles in patients with QFS. However, the results are doubtful, since some of them are based on the detection of *C. burnetii* by PCR (643, 644), suggesting rather persistent *C. burnetii* infection, and this hypothesis was not confirmed in the follow-up of patients in the UK (636).

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A more recent body of literature concerning QFS has been available since the Netherlands outbreak. Interest in this pathology was increased during and after the outbreak, not only in the scientific world. Thus, the real prevalence of this syndrome in this situation is difficult to evaluate. In an outbreak situation such as the one experienced in the Netherlands, sudden media coverage about a new disease can be stressful, and various information and rumors can easily circulate within social networks. As a consequence, and because primary Q fever can be asymptomatic, a higher proportion of patients having a great diversity of subjective symptoms could attribute their malaise to Q fever. For that reason, efforts have been made to use the most standardized tools possible for the diagnosis of QFS. The authors of the first studies used the Nijmegen Clinical Screening Instrument (NCSI) for assessment of the patient's health status. This is an empirically validated battery of tests evaluating functional impairment, subjective symptoms, and quality of life (645). In these studies, patients with possible, probable, or proven "chronic Q fever" were excluded. Limonard et al. investigated the health status of 82 patients with a past primary infection at 1 and 4 years of follow-up and found a higher frequency of severely impaired general quality of life and undue fatigue (50% and 46%, respectively) than in an adjusted control group (645, 646). These proportions were stable through the years. Morroy et al. identified 58.9% of patients with abnormal fatigue at 26 months of follow-up among 515 reported Q fever patients (647). Van Loenhout et al. performed a cohort study over a 24-month follow-up, using NCSI and SF-36 scores (648) among reported Q fever patients. They found rates of severe fatigue of 73% at 3 months, and this proportion decreased to 37% at 24 months but was still significantly higher than that in a healthy control group (2.5%). Baseline characteristics that were associated with long-term impaired health status were young women and preexisting health problems. In 2015, Keijmel et al. compared the characteristics of QFS patients and patients with CFS of other etiologies (649). QFS patients were significantly older, had a higher body mass index (BMI), and were more frequently men than patients with CFS. They also had received treatment for depression less often before the onset of symptoms. However, the two preceding studies suffer from a bias, which is the inclusion only of patients "reported" as having acute Q fever following the European guidelines (407), i.e., only patients with symptomatic primary infection. This bias was highlighted by Van Loenhout et al., who showed that long-term health status was altered at comparable levels for 193 reported and 448 nonreported cases at 4 years of follow-up (407).

MANAGEMENT STRATEGY FOR PATIENTS WITH *C. BURNETII* INFECTION

Given the clinical polymorphism of *C. burnetii* infection, there is no single management strategy. Recent studies have revealed that each situation requires specific treatment and follow-up. Regarding primary infection, the main issues after diagnosis are screening for potential risk factors for complications and choice of a prophylactic treatment with doxycycline (200 mg/day) and hydroxychloroquine (600 mg/day) to prevent progression to persistent focalized infection. Regarding endocarditis and vascular infections, early diagnosis should help to promptly initiate appropriate antibiotic therapy and rapidly decide if surgical treatment is needed. The special situation represented by pregnant women and children requires specific therapeutic options. In the case of other related complications such as chronic fatigue syndrome, no definite strategy is recommended to date, but cognitive behavioral therapy and doxycycline are under evaluation in a randomized controlled study in the Netherlands (650).

Treatment, Screening Strategy, and Follow-Up of Primary Infection

Treatment. When primary infection is symptomatic, it is recommended to initiate antibiotic treatment using doxycycline (200 mg per day). The first comparative nonrandomized study was performed in 1962 (651). Powell et al. compared two regimens of treatment and showed that duration of fever was shorter (1.7 days) in patients treated with doxycycline than in untreated patients (3.3 days) (651) This treatment seemed to be more effective when initiated within the first 3 days of symptoms.

Sobradillo et al. also observed that doxycycline treatment was associated with a more rapid decrease of fever (652). A recent retrospective study performed by Dijkstra et al. during the outbreak in the Netherlands confirmed that treatment with doxycycline, a fluoroquinolone, clarithromycin, or co-trimoxazole was associated with a reduced risk of hospitalization (653) compared with that for patients receiving beta-lactams or azithromycin. The same study found that a delay in treatment initiation of more than 7 days was associated with a higher rate of hospitalization. Side effects of doxycycline such as photosensitivity can be prevented by solar protection, and women should be on effective contraception for the duration of treatment. The standard duration of treatment is 14 days (654).

In case of doxycycline intolerance, minocycline, clarithromycin (500 mg twice daily), fluoroquinolones (ofloxacin 200 mg three times a day or pefloxacin 400 mg twice a day), and co-trimoxazole (160 mg trimethoprim and 800 mg sulfamethoxazole twice daily) are alternatives (17, 18). Azithromycin should not be a first choice, since *in vitro* studies have shown an elevated MIC (>8 mg/liter) and because of a higher risk of hospitalization with this treatment in the study from the Netherlands (21, 25, 653). For patients with neurological involvement during primary infection, fluoroquinolones are an interesting choice, since they have a good penetration in the cerebrospinal fluid (31). Glucocorticoids have been added to doxycycline in anecdotal reports, but we do not recommend using this treatment, since it can favor progression to persistent infection (292).

One study by Kampschreur et al. suggested that treatment of primary infection with doxycycline may prevent progression to persistent focalized infection (655). However, a significant bias of this result is that patients who have been treated for primary infection may have benefited from screening for risk factors and from closer follow-up than patients without treatment, who may have been actually undiagnosed during primary infection. To date, in the case of asymptomatic patients or when the diagnosis is made after resolution of symptoms, no treatment is recommended, except in the case of pregnancy (42).

Screening for risk factors of persistent focalized infection. After treatment of *C. burnetii* primary infection, the duration of treatment and follow-up are determined according to the results of the screening for risk factors of persistent infection. If these risk factors are detected, antibiotic prophylaxis with doxycycline and hydroxychloroquine should be initiated. This combination has proven to be effective in preventing endocarditis in a cohort study from our center (320). Since hydroxychloroquine may induce ocular side effects, ophthalmological examination before treatment and every 6 months during follow-up is recommended. Also, this drug is contraindicated in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency (654). When prophylaxis is initiated, we recommend serological follow-up 1 month after initiation of treatment and then every 3 months (422).

(i) Risk factors for endocarditis. As discussed in the preceding section, age and the presence of a valvulopathy are the most important risk factors for progression to *C. burnetii* endocarditis. Thus, we recommend performing systematic transthoracic echocardiography (TTE) in patients with primary infection. Antibiotic prophylaxis must be initiated in case of cardiac valve prosthesis, grade ≥2 valve stenosis or regurgitation, mitral valve prolapse, aortic bicuspidy, or remodeling or thickening of the valve (295) (Fig. 5).

F5

Million et al. also demonstrated in a cohort study that high levels of IgG aCL were significantly more frequent in patients with valvulopathy (295). Therefore, in *C. burnetii* primary infection, IgG aCL may be a biomarker of valvulopathy, as it was highly elevated in patients for whom valvulopathy was diagnosed after two TTEs. Moreover, they also observed that greatly elevated IgG aCL during primary infection was an independent predictor of progression to endocarditis. For this reason, we recommend including an assay of IgG aCL in the systematic screening of patients with *C. burnetii* primary infection. Age greater than 40 and IgM II levels of >3,200 were also associated with progression to endocarditis. Consequently, in patients >40 years with an IgG aCL level

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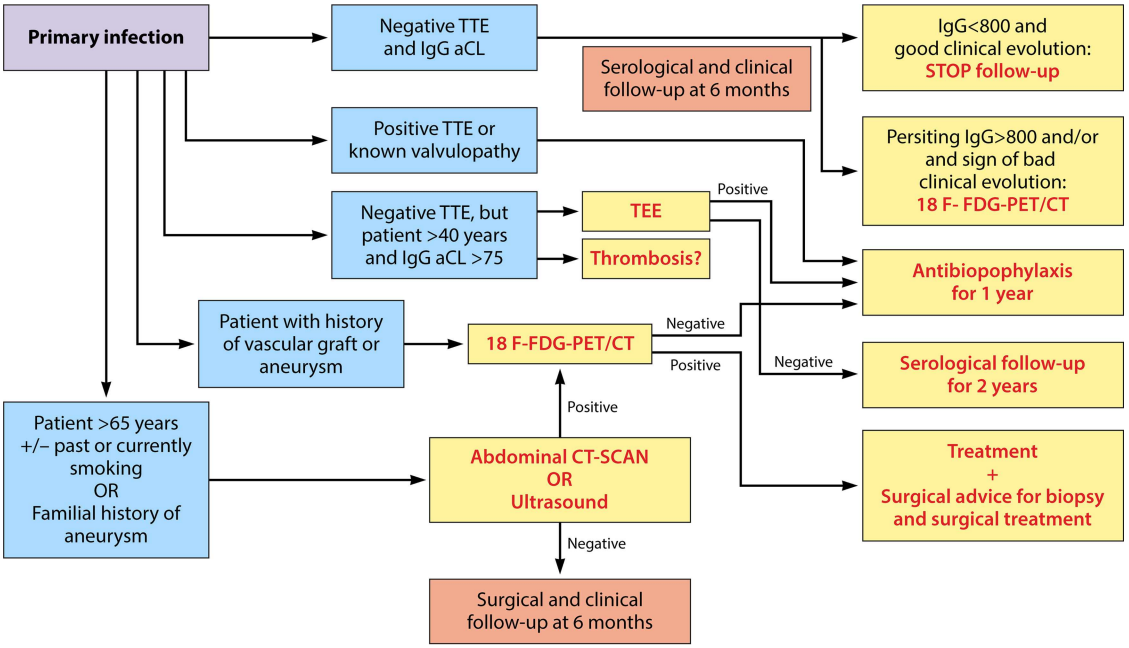

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FIG 5 Management strategy for *C. burnetii* infection.

of >75 IU and a normal TTE, we recommend performing transesophageal echocardiography (TEE) to rule out any valvulopathy (295) (Fig. 5). If the TEE is negative, we recommend close serological and clinical follow-up for 2 years. Conversely, if the TTE is negative in a patient with a low IgG aCL level, we recommend routine serological and clinical follow-up at 6 months. Finally, high IgG aCL levels have been reported in patients presenting with thrombosis during *C. burnetii* primary infection (532, 533; Million et al., submitted). Consequently, we suggest screening patients for thrombosis in cases of high IgG aCL, in particular for thrombophlebitis of the leg and pulmonary embolism. (Fig. 5). In this context, in addition to doxycycline, patients with high IgG aCL levels (≥75 GPLU) may benefit from hydroxychloroquine treatment until the IgG aCL decreases to under this value (Million et al., submitted). Patients with thrombosis history, procoagulable state, or exceptionally high IgG aCL levels require prophylactic anticoagulation.

(ii) Risk factors for vascular infections. *C. burnetii* vascular infections are very severe, and no prophylactic strategy existed until recently. Major risk factors are vascular grafts and the presence of preexisting vascular aneurysms. The most frequent localization is the abdominal aorta (418). In a recent study, we proposed a screening strategy to detect patients with undiscovered abdominal aortic aneurysm (418). Since age of >65, tobacco use, and familial history of aneurysm are the main risk factors for vascular aneurysms, we recommend performing an abdominal CT scan or ultrasound (in case of renal contraindication) in these patients. If a vascular aneurysm is detected, we recommend performing 18 F-FDG PET/CT to screen for signs of early infection. In case of a negative 18 F-FDG PET/CT, initiation of doxycycline and hydroxychloroquine should be done for prophylaxis (Fig. 5).

(iii) Immunocompromised hosts. For immunocompromised patients, no data are available in the literature in favor of prophylaxis. However, we recommend close serological monitoring of these patients at 3, 6, 9, and 12 months after primary infection, because they may be at higher risk for developing focalized persistent infection (654).

(iv) Follow-up strategy when no risk factor is detected during primary infection. When all screening tests are negative, routine serological and clinical follow-up should be performed at 6 months (654). If serological titers persist at levels higher than 800 at 6 months of follow-up and if the patient shows signs of a poor clinical outcome,

(persistent fever, asthenia, weight loss, or persistent superficial adenopathy), an 18 F-FDG PET/CT scan should be performed. In a recent study, we showed that this exam discovered an unsuspected focus of *C. burnetii* infection in 38.7% of patients with persistent IgG I titers of >800 (412).

Treatment and Follow-Up of Persistent Focalized Infection

Treatment and follow-up of endocarditis. Antibiotic treatment for *C. burnetii* endocarditis combines doxycycline (200 mg/day) with hydroxychloroquine (200 mg 3 times/day). Hydroxychloroquine is necessary to raise the pH in the pseudolysosomal vacuole to restore doxycycline activity (36, 656). This combination has shown bactericidal activity *in vitro* (36). In 2010, Million et al. reported the results of a survey of 104 patients with *C. burnetii* endocarditis followed in our center (415). The main side effects reported were photosensitization in 23% of patients, digestive intolerance in 7%, ocular toxicity in 4%, and irreversible skin pigmentation in 3% (415). In that study, the rate of endocarditis-related mortality was 4% after 3 years of follow-up. Independent factors associated with death were age at diagnosis, stroke at diagnosis, prosthetic valve, and absence of a 4-fold decrease in IgG and IgA at 1 year of follow-up. The ideal time for serological cure (IgG1 level of <800) was 41 months, and 36% of patients had serological cure at 3 years. Independent factors associated with serological failure were male sex, the presence of IgG1 at diagnosis, and delay in treatment initiation. Forty-five percent of patients had surgical treatment, which was more frequently performed in cases of heart failure and cardiac abscess. However, surgery was not associated with a better survival, except in the group of patients with a valvular prosthesis. Clinical and serological cure is possible without valve replacement. Analyses of the excised valves (by culture and IHC) were all negative after 24 months of treatment. However, two prosthetic valves were positive after 18 months. In addition, serological relapse was associated with treatment of less than 18 months and prosthetic valve endocarditis. For these reasons, we recommend treating patients with native valve endocarditis for 18 months and patients with prosthetic valve endocarditis for 24 months. Longer treatment can be proposed in the case of absence of a 4-fold decrease in IgG and IgA and no disappearance of IgM II. Serological monitoring should be performed every 3 months during treatment. Rolain et al. have shown that serum doxycycline concentrations up to 5 μg/ml are correlated with good serological progression in patients with endocarditis (657). Thus, monitoring of doxycycline and hydroxychloroquine concentrations, with objectives of >5 μg/ml and 1 $\pm$ 0.2 g/ml, respectively, is useful. We observed 6% of patients with serological relapse at 5 years (415). For this reason, we recommend continuing serological monitoring until 5 years of follow-up.

Treatment and follow-up of vascular infections. For years no consensual guidelines were available concerning vascular infections, due to a too-small number of cases per center. However, a retrospective study of 32 patients by Botelho-Nevers et al. in 2007 suggested that surgical resection of the infected tissues was associated with recovery (417). Twenty-five percent of patients from this cohort had died at 3 years follow-up, and patients who died had a significantly shorter course of treatment (mean duration of treatment, 10 months), less frequent surgical treatment, and more frequent vascular rupture. Therefore, doxycycline and hydroxychloroquine treatment should last for a minimum of 24 months.

More recently, we performed a retrospective study of all patients with infections of vascular aneurysms or grafts who have been followed in our center between 1986 and 2015 (418). For 66 patients, a follow-up of 2.5 years was available, and the mortality rate was 18.5%. Mortality rates were significantly different for patients who were operated on and those who were not (6.5% versus 28.6%, respectively; $P = 0.02$), and surgical treatment was the only independent factor associated with survival (418). Surgical treatment was also a predictor of good serological progression. For three patients, qPCR on a vascular biopsy specimen was positive after 2 years of treatment, and for one patient, culture was also positive at that time. This illustrates that antibiotic treatment

alone is not sufficient to eradicate the infection in this setting and that resection of the infected tissue is mandatory, due to the high bacterial load.

In our study, patients with vascular graft infection were less frequently operated on than patients with vascular aneurysm infections, and in this subgroup of patients, surgery was associated with good serological progression, but correlation with survival was not statistically significant. This lack of correlation may be due either to a lack of statistical power or to the fact that postoperative mortality was higher in the group of patients with vascular grafts. In fact, these patients may have more cardiovascular comorbidities and higher anesthesia risks than patients with infection of vascular aneurysms.

The conclusion from this study is that surgical treatment should be performed in the case of *C. burnetii* infection of a vascular aneurysm. In the case of patients with vascular grafts, surgery should be performed after careful assessment of surgical and anesthesia risks. Semiconservative surgery, which was successful in a case report from Kloppenburg et al., may be a solution in patients presenting a very high surgical risk (658).

Serological monitoring should follow the same pattern as for endocarditis.

Treatment and follow-up of other persistent focalized infections. For other types of persistent focalized infections, such as osteoarticular infections and persistent lymphadenitis, no cohort study is available, so that treatment options rely on case reports and expert advice.

Regarding osteoarticular infection, administration of doxycycline and hydroxychloroquine for 18 months has been reported to be the best option (419, 420). However, one case of treatment failure was reported in a patient with prosthetic joint arthritis, but this patient had rheumatoid arthritis and was on immunosuppressive treatment. In this case, surgical removal of the prosthesis was necessary for cure. To date, no treatment recommendation exists for persistent lymphadenitis. We propose the same 18-month duration of antibiotic treatment when this diagnosis is definite according to our recent criteria. The same serological monitoring as for endocarditis and vascular infections should be performed in such patients.

Treatment and Follow-Up of Pregnant Women and Children

Pregnant women. A retrospective cohort study compared obstetrical outcomes in 16 pregnant women who received long-term co-trimoxazole (i.e., 160/800 twice daily for 5 weeks) versus 37 who did not receive this treatment. Obstetrical complications were observed in 81.1% of pregnant women without treatment versus 43.9% of pregnant women who received long-term co-trimoxazole ($P = 0.009$) (659). In particular, no intrauterine fetal deaths were observed in the latter case. Moreover, this treatment reduced the rate of placentitis and progression to a chronic serological profile. Angelakis et al. have confirmed this result, with 42% of treated pregnant women presenting obstetrical complications versus 100% of untreated pregnant women (164). Finally, a meta-analysis by Million et al. on this subject included 136 pregnancies (4 case histories and 7 population-based studies). This study revealed an increased risk of fetal death when *C. burnetii* serology was positive, but this risk was significantly reduced when pregnant women were treated (148). Because co-trimoxazole is a folic acid antagonist, supplementation with folic acid should be administered during the first trimester of pregnancy. After treatment, close serological follow-up should be performed at 3, 5, 9, 12, 18, and 24 months for early detection of progression to persistent focalized infection.

When persistent focalized infection is diagnosed during pregnancy, co-trimoxazole should be initiated and maintained until delivery. After delivery, a change to doxycycline and hydroxychloroquine should be made. In case of diagnosis in the peripartum period, breastfeeding is not recommended, due to possible transmission to the newborn (659).

Children. Doxycycline is used in the treatment of primary infection for children >8 years old (310, 609, 612). The pediatric dose for doxycycline is 2.2 mg/kg twice daily (42). The duration of treatment is 14 days, as for adults. For children with mild primary

infection who are younger than 8 years old, we recommend using co-trimoxazole because of the risk of dental staining. However, a recent review by Cross et al. concerning the risks and benefits of doxycycline suggests that, conversely to the case for tetracycline, no strong evidence of correlation with dental staining and teratogenic effects can be found in the literature (660). Therefore, in children <8 years old with severe primary infection, we recommend using doxycycline.

Regarding the treatment of persistent focalized infection in children, few data are available in the literature. Cases of endocarditis and infection of a bovine jugular vein conduit were treated with doxycycline plus hydroxychloroquine and surgery (187, 616). These cases involved children 11 and 13 years old. Osteomyelitis is treated with variable combinations of the following antibiotics: co-trimoxazole, ciprofloxacin, rifampin, doxycycline, clarithromycin, and azithromycin (578, 581, 582). The most frequent duration of treatment found in the literature is 6 months, and surgical drainage of multifocal osteomyelitis is often needed for cure (578). One reference laboratory described successful surgical treatment alone in a child with a single bone lesion (579). An anecdotal case of treatment with gamma interferon as salvage therapy in a 3-year-old child is reported, but it does not seem reasonable to recommend this treatment (661).

Treatment of Chronic Fatigue Syndrome

Regarding the treatment of QFS, a prospective randomized trial is currently in progress in the Netherlands to compare the efficacy of doxycycline and cognitive behavioral therapy versus placebo (650). Inclusion criteria for this study are based on the definition of Q fever fatigue syndrome (QFS) from the Dutch National Consensus. Cognitive behavioral therapies have proven to be effective in patients with chronic fatigue syndrome following other diseases and may therefore be an interesting option.

Prevention

In some situations, Q fever is an occupational disease. This has been illustrated by the first description of the disease, which occurred in a population of slaughterhouse workers. Also, a member of a laboratory team cultivating *C. burnetii* in the 1930s was infected (375). The main categories of people occupationally exposed are those working with animals (farmers, slaughterhouse workers, and veterinarians) and people working in laboratories cultivating the bacterium. Cases involving medical staff consist of people who participated in autopsies of patients with Q fever (662, 663) or an obstetrician who managed parturient women with Q fever (130). Cases in the military have also been reported (1, 664, 665). In this section we detail the available prophylactic measures in occupational settings.

Vaccination. A vaccine has been available in Australia since 1989 (Q-Vax; CSL Biotherapies, Parkville, Victoria, Australia) (666). It is a whole-cell formalin-inactivated vaccine produced and licensed in Australia. Its efficacy has been tested in one randomized control study among 200 slaughterhouse workers. During 15 months of follow-up, there were seven cases in the control group and no cases in the vaccinated group (667). However, this vaccine can induce local reactions, and patients should be evaluated with a cutaneous test (Q-Vax skin test) for Q fever before vaccination to avoid severe side effects. The Australian Veterinary Association (AVA) recommends vaccination for all veterinarians, veterinary students, and veterinary nurses. A recent survey in that country has shown that 74% of veterinarians and 29% of veterinary nurses sought vaccination (666). Also, a nationally funded vaccination program was initiated in the country in 2002 (668). Program adherence was 100% among slaughterhouse workers and 43% in farmers. After this campaign, reporting for Q fever decreased by 50%, and the number of hospitalizations also decreased (668). In 2011, in the southeast of the Netherlands, a vaccination campaign targeted people at risk for progression to endocarditis and vascular infections during the epidemic (669). However, coverage rates in high-risk people of only 11% to 18% were observed, illustrating poor efficacy from a public health perspective. No other country has tried to institute a large-scale vaccination program among occupationally exposed persons to date. In these areas, the

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improvement of clinicians' awareness about the risk for *C. burnetii* infection in some categories of workers should help in the early diagnosis and treatment of the infection.

Isolation. Regarding the prevention of transmission in laboratory workers handling *C. burnetii* cultures, all manipulations have to be made in a biosafety level 3 (BSL3) laboratory with appropriate personal protective equipment (PPE). For health care personnel performing autopsies on patients suspected to have died from Q fever, wearing of an N95 respiratory protection mask is recommended. The same recommendation can be made for obstetrical staff who are in contact with parturient women diagnosed with *C. burnetii* infection.

Moreover, because *C. burnetii* can survive in the environment in a spore-like form, contaminated surfaces should be cleaned with a solution containing a dual quaternary ammonium-detergent compound, which completely inactivates the bacteria after a 30-min contact time (42). A 1:100 dilution of household bleach is also an effective solution.

PERSPECTIVES AND FUTURE CHALLENGES

A more accurate assessment of the risk factors in the progression to *C. burnetii* endocarditis will require particular studies. The understanding of individual susceptibility factors will allow better management through active treatment in order to prevent progression to this still-severe disease. The systematic detection of *C. burnetii* by multiplex PCR strategies in syndromes such as endocarditis, pneumonia, hepatitis, and fever during pregnancy should allow diagnosis and treatment of more cases in the world. Finally, new therapeutic strategies with shorter courses and better tolerance should be developed for endocarditis and vascular infections. *In vitro* testing, new antibiotic combinations, and randomized studies comparing new protocols to the therapeutic approach that has been used for the last 20 years are necessary and will be a major subject of future research.

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Max Maurin, M.D., Ph.D., is 54 years old and trained in Professor Didier Raoult's team from 1991 to 2002. He received his M.D. degree at the Medical Faculty of Marseille, Aix-Marseille II University, France, in 1991 and his Ph.D. degree at the Bichat Faculty, Paris VII University, France, in 1993. He was appointed as a professor of bacteriology at Grenoble University Hospital (Grenoble Alpes University) in 2002. He has been the head of this bacteriology laboratory since 2002 and of the French national reference Center for *Francisella* since 2006. Since 1991, he has been involved in the study of strict and facultative intracellular bacteria that are pathogenic for humans and animals. His main research topic has been focused on the characterization interactions between intracellular bacteria and antibiotics.

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Didier Raoult, M.D., Ph.D., specializes in infectious diseases and is a professor of microbiology at the Faculty of Medicine of Marseille, Aix Marseille University. In 1984, he created *ex nihilo* his research laboratory, the Rickettsia Unit. This unit has now become the Research Unit in Infectious and Tropical Emergent Diseases (URMITE), collaborating with the CNRS (National Center for the Scientific Research), the IRD (Institute of Research for Development), and INSERM (National Institute of Health and Medical Research). In 2011, he became the director of the University Hospital Institute Mediterranée Infection, which is a 600-person medical institute focused on infectious diseases. This facility includes the largest diagnostic and research microbiology laboratory in France. As of 2014, Professor Raoult has published more than 1,900 indexed publications. In the last 30 years, he has cultured approximately 16% of the bacteria isolated for the first time in humans, including *Tropheryma whipplei*, in his laboratory, and he developed a culturomics team in 2011.

Partie II : Optimisation du milieu de culture axénique ACCM2 par ajout d'une molécule antioixydante

AVANT-PROPOS

L'élaboration d'un milieu de culture axénique pour *C. burnetii* représente une révolution dans l'étude de cette bactérie. Néanmoins, l'un des inconvénients de la culture sur milieu axénique ACCM2 est la nécessité d'obtenir une atmosphère microaérophile (2.5% d'oxygène) ce qui sous-tend l'utilisation d'incubateurs spécifiques et représente donc une contrainte technique. Notre équipe a démontré précédemment que l'ajout de molécules antioxydantes (acide urique, acide ascorbique ou glutathion) permettait pour un certain nombre de bactéries considérées comme anaérobies, d'obtenir une croissance en milieu aérobie (9). Nous avons donc testé l'ajout d'acide urique dans le milieu ACCM2 à différentes concentrations. Les concentrations bactériennes dans les différents milieux ont été évaluées par microscopie optique au grossissement X100 après coloration de Gimenez. Nous avons ainsi comparé la croissance axénique de la souche Nine Mile de phase II de *C. burnetii* dans les conditions suivantes: (1) Milieu ACCM2 incubé en atmosphère microaérophile (2) Milieu ACCM2 incubé en atmosphère aérobie sans acide urique (3) Milieu ACCM2 avec acide urique (à des concentrations de 0,5 à 1 microgr/ml) en atmosphère aérobie.

Nous avons observé une croissance d'environ 3 log à J6 pour le milieu ACCM2 incubé en conditions microaérophiles et des microcolonies ont pu être observées sur gélose. En conditions aérobie sans acide urique, une croissance d'environ 1 log était observée à J8 et une fine pellicule était observée à a surface de la gélose sans réelles microcolonies visualisées. En ce qui concerne le milieu ACCM2 supplémenté par de l'acide urique à des concentrations de 0,5 à 1 microgr/ml, nous avons observé une croissance intermédiaire de l'ordre de 2 à 2.5 log à J8, avec apparition de microcolonies sur la gélose.

Nous avons donc réussi à optimiser les conditions de culture axénique de *C. burnetii*, en montrant qu'il était possible de cultiver la bactérie en atmosphère aérobie grâce à l'ajout d'acide urique. Néanmoins, ces résultats ont été obtenus avec des inocula purifiés à de fortes

concentrations et la reproductibilité de ces résultats doit donc être testée avec des inocula à de plus faibles concentrations, afin d'améliorer la sensibilité de cette technique, notamment dans le but d'isoler *C. burnetii* à partir d'échantillons cliniques.

Article 2: Addition of Uric Acid in ACCM2 facilitates *C. burnetii* axenic aerobic growth

En préparation

Addition of Uric Acid in ACCM2 facilitates *C. burnetii* axenic aerobic growth

Carole Eldin and Didier Raoult

Introduction

C. burnetii is an intracellular bacterium that causes an acute primary infection called "Q fever" and localized persistent infection like endocarditis, vascular or osteoarticular infections(1). This microorganism has long been considered as a strict intracellular bacterium, making its isolation fastidious and time consuming due to cultivation on cell cultures.

Recently, two axenic media for the culture of *C. burnetii* have been published (2, 3). One medium is developed by our team and is called the Vero Cell Extract Medium (VCEM) (2). To date, this medium allows the growth of *C. burnetii* only in liquid conditions and adding fresh medium every 48 hours is necessary to sustain growth. Omsland *et al.* elaborated another axenic medium, called the Acidified Cysteine Citrate Medium 2 (ACCM2), which is composed with an acidic citrate buffer, neopeptone, fetal bovine serum, RPMI medium and methyl-β-cyclodextrin (3). This medium has an acidic pH (4,5) and is incubated in 2.5% oxygen. It allows a 4 to 5 log growth at day 7 of culture. The authors had previously shown that *C. burnetii* was a microaerophilic organism, with a maximal oxidation of metabolic substrates when incubated in 2.5% Oxygen atmosphere (4). The microaerophilic incubation is a technical constraint in the use of ACCM2 because specific incubators are needed.

Recently, our team has demonstrated that some microorganisms that were considered strictly anaerobic could be cultivated aerobically thanks to the adding of antioxidant compounds to the medium (ascorbic acid, glutathione and uric acid of culture medium with)(5–7). Uric acid has an antioxidant activity under certain conditions and has been shown to enable the growth of eight anaerobic species under aerobic conditions in a recent report (6).

We tested if the addition of uric acid in ACCM2 could allow the growth of *C. burnetii* in aerobic conditions, which could simplify its axenic culture.

Material and Methods

Inoculum preparation

For this study we used the avirulent Nine Mile phase II strain. *C. burnetii* strains were cultivated in confluent Vero cells (African green monkey kidney cells) grown in Minimum Essential Medium (MEM) (Life Technologies) supplemented with 4% fetal bovine serum (FBS) (Life Technologies) and 1% L-glutamine (Life Technologies,) at 37°C in the presence of 5% CO2. Purification of bacteria was done thanks to adjunction of glass beads and successive centrifugations, as previously described (2). The amount of inoculum was normalized by counting bacteria using Gimenez staining. A conversion factor was established based on the number of bacteria observed per field by light microscopy at *X 100* magnification. The concentration of bacteria was determined using the assumption that one bacterium observed per field reflects a population of 10^4 bacteria/ml, as previously described (2).

ACCM2 and Uric Acid

ACCM2 liquid medium was prepared as described by Omsland *et al.* (3). For solid medium preparation, we added agarose but without the thin overlay that was used in the initial description of the medium. Plates at concentration of 0.25% agarose were prepared.
Uric acid (Sigma-Aldrich) was added to ACCM2 at the following final concentrations: 0.5 mg/mL and 1 mg/mL.

Inoculation

Nine Mile Phase II inoculum at a concentration of 10^6 bacteria/ mL was diluted in a ten-fold volume of liquid ACCM2 with or without uric acid. For solid medium inoculation,

200 µl of inoculum was spread on the surface of ACCM2 agarose plates thanks to an oese. ACCM2 with and without uric acid, inoculated with C. burnetii Nine Mile phase II strain was incubated in a 2.5% oxygen environment or in aerobic conditions.

Quantification of growth

Growth quantification was performed after a minimum of 5 days of incubation in liquid and solid ACCM2. Quantification was performed thanks to Gimenez staining of a sample of 50 µl of medium and microscopic examination at *X 100* magnification following the quantification method described above. For solid media, macroscopic examination of plates was performed each day and Gimenez staining was done in a sample of microcolony to be sure that there was no contamination of the medium by others bacteria.

Each condition of growth was performed in triplicate.

Results

When *C. burnetii* was cultivated on ACCM2 with 2.5% oxygen, we observed a growth of about 3 log at day 6 (from log 5 to 7.69, Figure 1A and 1B). In solid ACCM2 with 0.25 % agarose, microcolonies were observed from day 6 of growth and Gimenez staining confirmed the presence of *C. burnetii*. (Figure 1A and 1B). When liquid ACCM2 was incubated in aerobic conditions, a 1 log-growth was observed at day 8 (Figure 1B, Figure 2). In solid ACCM2 in aerobic conditions, a thin layer on the surface of the plate was observed in raking light at day 7 (Figure 1B) and Gimenez staining was positive. In liquid ACCM2 with 0.5 mg/mL of uric acid, a 2 log growth was observed at day 8 (Figure 1A and B) and microcolonies were observed in solid medium plates at day 7. (Figure 1B). When the dosing of uric acid was increased at 1mg/mL, the growth was higher (2.5 log) at day 8 (Figure 1A). In solid medium, microcolonies were observed at day 7 and Gimenez staining was positive (Figure 1B)

Discussion

The first result of our study is that we observed a 2.7-log growth of *C. burnetii* Nine Mile phase II in ACCM2 incubated in microaerophilic conditions. Omsland *et al.* obtained a 4 to 5 log-growth by adding a shaking of the samples in culture (3), which we didn't chose to do in this work. Without shaking, the same range of growth (around 3 log) was observed in their previous studies (4). The second result is that growth was also observed in ACCM2 incubated in aerobic conditions, which was quite unexpected given the results of Omsland *et al.* who observed no growth in 20% oxygen incubation (4). However, they obtained this result with the first version of the medium, called ACCM, which was then improved with adjunction of methyl-β-Cyclodextrin. Nevertheless, in our study, the growth observed in aerobic conditions was much less important than in microaerophilic conditions (around 1 log vs 2.7log).

Our main result is that adjunction of uric acid to ACCM2 led to restore 1 log of growth in aerobic conditions at dosing of 0.5 mg/mL and 1 mg/mL (Figure 1A and 1B). This antioxidant molecule has already proven its usefulness in the culture of several anaerobic bacteria (6). This finding is a new step in the facilitation of *C. burnetii* axenic cultivation.

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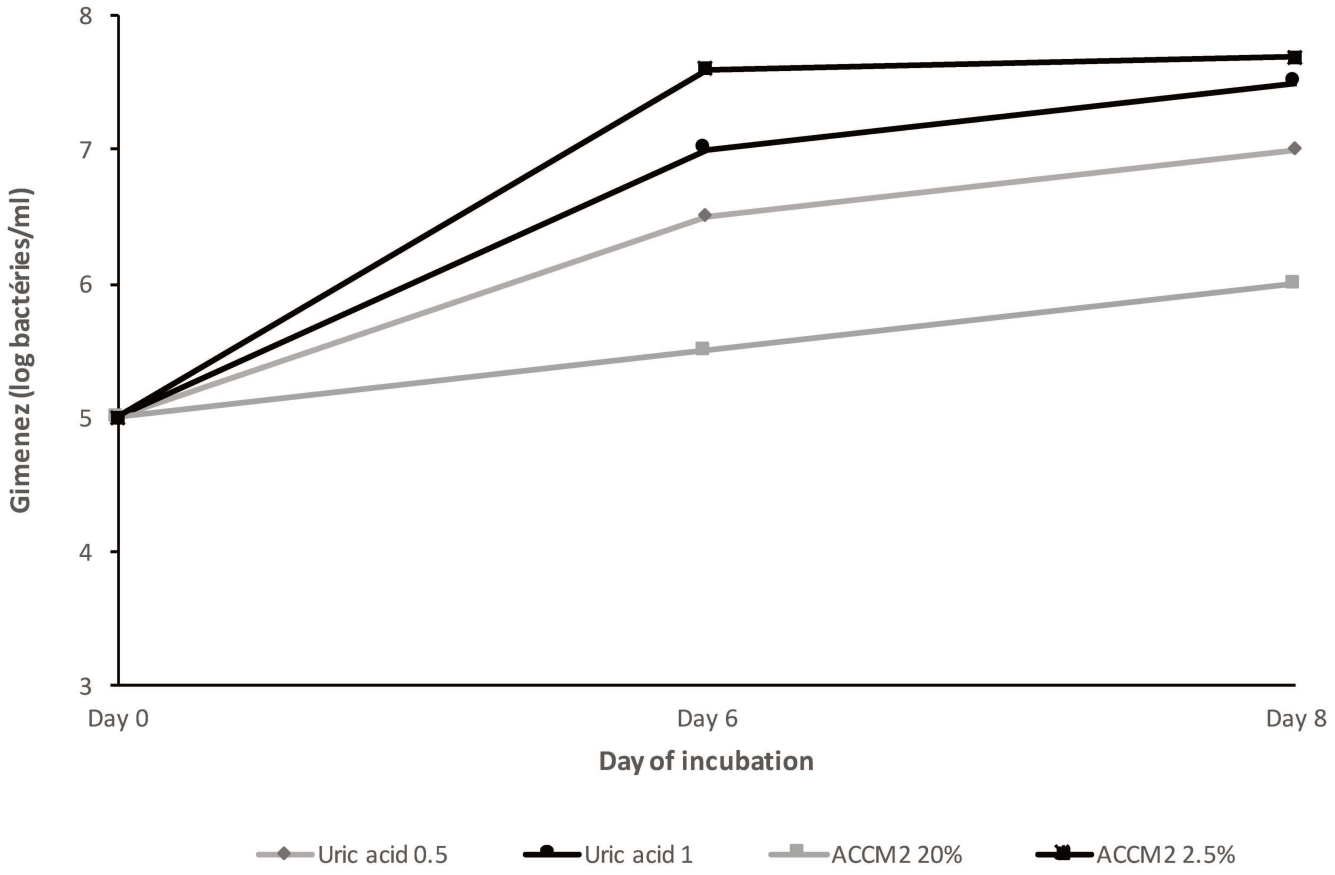
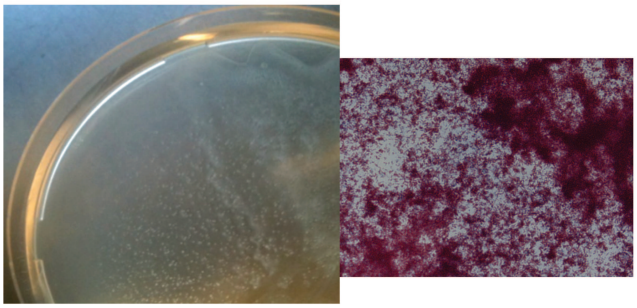
Figure 1. Comparison of growth

A. Liquid Medium

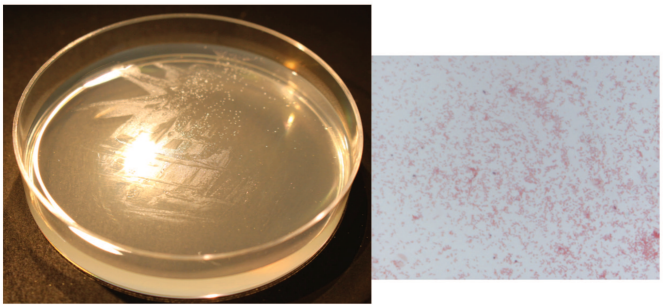


B. Solid Medium at Day 7 (Macroscopic and Gimenez)

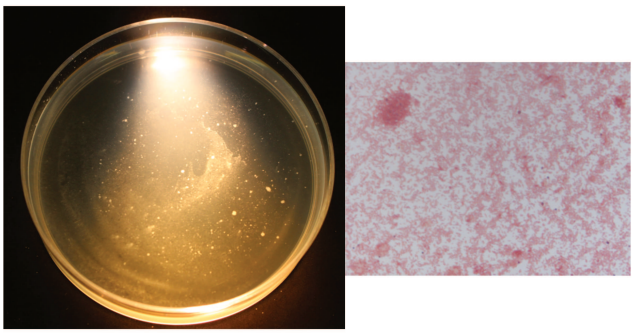
ACCM2 2.5% O2



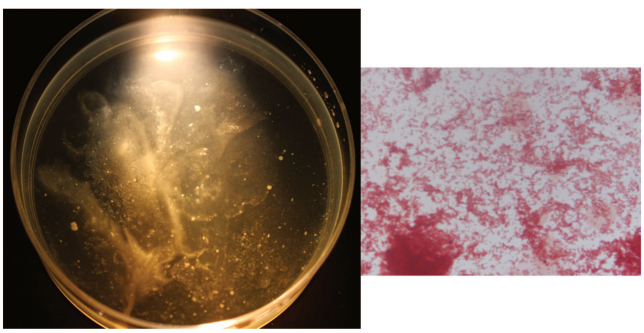
ACCM2 20 % O2



ACCM2 + Uric Acid (0.5)



ACCM2 + Uric Acid (1)



Partie III : Virulence, génome et sensibilité aux antibiotiques de *Coxiella burnetii* MST 17, les souches isolées à Cayenne, Guyane Française.

AVANT-PROPOS

A Cayenne en Guyane Française, l'épidémiologie de la fièvre Q présente des caractéristiques exceptionnelles. La prévalence de l'infection est la plus élevée au monde puisque 24% des pneumopathies communautaires sont causées par *C. burnetii*, ce qui en fait une zone hyperendémique (8). La manifestation clinique principale de la primo infection à *C. burnetii* dans cette zone est la pneumopathie (10). Pour cette raison, l'antibiothérapie probabiliste des pneumopathies communautaires à l'hôpital de Cayenne comprend une beta-lactamine associée à de la doxycycline afin de couvrir cette bactérie intracellulaire. L'augmentation de l'incidence de la maladie semble avoir débuté dans les années 90, quand trois patients ont présenté une pneumopathie avec détresse respiratoire entraînant le décès de l'un de ces cas. Ceci reflète le fait que ces pneumopathies ont une présentation clinique plus sévère que les pneumopathies causées par d'autres germes à Cayenne. En effet, une étude récente a montré que les patients atteints de fièvre Q présentaient plus fréquemment des frissons, des sueurs nocturnes, des céphalées ou des myalgies (10). Sur le plan biologique, les patients atteints de fièvre Q à Cayenne présentent également des niveaux de CRP plus élevés. L'ensemble de ces caractéristiques font penser que la souche responsable de la fièvre Q à Cayenne est hypervirulente. Or, il a été montré que l'ensemble des souches isolées à partir de patients de Cayenne appartenaient au même génotype (MST 17). Après avoir effectué une revue de la littérature sur l'apport des outils de la génomique dans l'étude de *C. burnetii,* nous avons travaillé sur deux axes concernant ces souches.

Tout d'abord, nous avons pris part au travail de séquençage d'une souche provenant de Cayenne, la souche Cb175. Cette souche avait été isolée en 2012 à partir d'une valve cardiaque d'un patient de 60 ans qui présentait un tableau d'endocardite à *C. burnetii.* Après une étape de culture et de purification de la souche, le séquençage a été effectué par la méthode SOLiD. Durant l'étape de « finishing », une délétion de 6105 paires de bases a été mise en

évidence, en comparaison avec le génome de la souche de référence Nine Mile. Une PCR a donc été élaboré ciblant la délétion, afin de voir si celle-ci était présente dans les autres souches isolées à Cayenne, ainsi que dans les souches de *C. burnetii* disponibles au laboratoire. Ceci a permis d'identifier que la délétion était présente chez les 8 souches de génotype MST 17 isolées à Cayenne et sur aucune des 298 souches de *C. burnetii* appartenant à d'autres génotypes ($p < 0.000001$). Cette délétion est localisée au niveau de l'opéron hlyCABD, qui intéresse le Système de Sécrétion de Type 1 (SST1) et occasionne une importante réduction de génome en comparaison à toutes les autres souches séquencées.

La réduction de génome est un phénomène observé chez plusieurs espèces bactériennes hypervirulentes (11). Plus particulièrement, la perte de gènes impliqués dans les systèmes de sécrétion a été observés chez les bactéries hypervirulentes. En effet, ces bactéries vont utiliser de façon préférentielles les systèmes de sécrétions de l'hôte qu'elles infectent, entraînant ainsi une pathogénicité plus importante. Dans le cas des souches de *C. burnetii* isolées à Cayenne, il est fort probable que cette réduction génomique impliquant le SST1 soit l'une des clés de l'épidémiologie exceptionnelle de la maladie sur ce territoire.

Nous avons dans un autre travail sur les souches de Cayenne, déterminé le profil de sensibilité aux antibiotiques de ces souches. En effet, au vu de la prévalence de la maladie, il est indispensable de savoir si les molécules actuellement prescrites à Cayenne sont réellement efficaces dans le contrôle de l'infection. Nous avons cultivé 6 souches de *C. burnetii* isolées à partir de patients de Cayenne sur cellules HEL en présence ou en absence des antibiotiques suivants: Doxycycline, minocycline, levofloxacine, sulfamethoxazole-trimethoprime, rifampicine, erythromycine, tigecycline, telithromycine et azithromycine. La croissance à été évaluée à J15 pour toutes les conditions de culture. Nous avons utilisé une méthode de quantification de la croissance originale, à savoir la cytométrie de flux. En effet, cette technique a pour avantage de détecter de façon spécifique *C. burnetii*, grâce à l'utilisation d'anticorps

marqués et aux caractéristiques de taille et de structure permettant de différencier la bactérie des débris cellulaires sur lesquelles elle est cultivée.

Toutes les souches étaient sensibles à la doxycycline et à la minocycline. Le suflaméthoxazole-trimetoprime était également actif sur toutes les souches et représente donc une molécule de choix pour le traitement des infections à *C. burnetii* chez les femmes enceintes à Cayenne, situation dans laquelle la doxycycline est contre-indiquée. En revanche, les 6 souches étaient résistantes à l'érythromycine et à l'azithromycine, et l'une d'entre elles était également résistante à la télithromycine, ce qui n'avait jamais été retrouvé auparavant chez *C. burnetii*. Ce travail permet de conclure que la doxycycline reste la molécule de choix dans le traitement des pneumopathies à *C. burnetii* à Cayenne, et que les macrolides ne devraient pas être utilisés de manière probabiliste dans cette indication.

Article 3 : The contribution of Genomics to the study of Q fever

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REVIEW

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The contribution of genomics to the study of Q fever

Felicetta D'Amato[1], Carole Eldin[1] & Didier Raoult*[,1]

Coxiella burnetii is the etiological agent of Q fever, a worldwide zoonosis that can result in large outbreaks. The birth of genomics and sequencing of *C. burnetii* strains has revolutionized many fields of study of this infection. Accurate genotyping methods and comparative genomic analysis have enabled description of the diversity of strains around the world and their link with pathogenicity. Genomics has also permitted the development of qPCR tools and axenic culture medium, facilitating the diagnosis of Q fever. Moreover, several pathophysiological mechanisms can now be predicted and therapeutic strategies can be determined thanks to *in silico* genome analysis. An extensive pan-genomic analysis will allow for a comprehensive view of the clonal diversity of *C. burnetii* and its link with virulence.

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KEYWORDS
• *Coxiella burnetii* • genomics
• pan-genome • Q fever
• virulence

C. burnetii is the agent of Q fever, a worldwide zoonosis. It is a small, pleomorphic, obligate intracellular Gram-negative bacterium [1]. The primary reservoirs of this zoonosis are cattle, goats or sheep that shed microorganisms in urine, feces, milk and birth products. Inhalation of contaminated aerosols represents the principal route of transmission to humans [1]. Genomics has emerged in Q fever studies since the first genome sequence of a *C. burnetii* strain was conducted in 2003 [2]. This breakthrough had major implications in all fields of study of this fastidious microorganism. Crucial domains, such as phylogeny, epidemiology, diagnostic, culture methods, pathophysiology, virulence factors and antibiotic resistance analysis, have benefited from this development. Until recently, the strict intracellular nature and high infectivity of *C. burnetii*, requiring a biosafety level 3 laboratory, has considerably complicated the study of its pathophysiology and mechanisms of virulence. The use of classical molecular-genetic tools and genetic transformations was fastidious and time consuming [3]. The elaboration of an axenic medium to cultivate *C. burnetii* was a major issue that has been significantly accelerated by the availability of a genome sequence. Omsland *et al.* performed *in silico* the prediction of the main metabolic pathways and the identification of the growth requirements of the bacterium from genome analysis [4]. This was the key step forward, following multiple combinations of culture conditions, culminating in an efficient axenic medium called acidified cystein citrate medium 2 (ACCM2) [5]. This major advance for those working on *C. burnetii* is the first illustration of the contribution of genomics to the study of Q fever.

Q fever is characterized by a wide clinical picture [1]. During the phase of primo infection, the infection can range from an asymptomatic seroconversion to symptomatic 'acute Q fever,' leading to severe pneumonia and death by respiratory distress syndrome [6]. Long-term complications of the infection are not linked to the severity of the primo-infection and may be observed after asymptomatic

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primo infection (not acute Q fever) [7]. When the infection persists, it can lead to endocarditis [8], vascular infections [9], osteoarticular infections [10] or persistent lymphadenitis [11] that can result in lymphoma. These infections were initially described as 'chronic Q fever,' a nomenclature that appears obsolete because it covers very different foci of infection with different treatment and prognosis. Moreover, 'chronic fatigue' is another consequence of Q fever. The prognosis of vascular infections is much more severe than that of *C. burnetii* endocarditis [8,9]. To date, no death has been reported from osteoarticular infections [10]. Conversely, lymphoma due to persistent infection is a severe disease [11]. Thus, as in the case of tuberculosis where precision of the localization is of importance, the localization of *C. burnetii* persistent infection should be actively searched and included in the unclear 'chronic Q fever' term. Chronic fatigue syndrome is a long-term complication without focus of infection that should be considered separately [12].

Historically, the principal factors identified to explain the clinical variability and outcome of Q fever were patient susceptibilities. Before the genomics era, the age and sex of patients were the first variables identified as affecting the clinical expression of the acute form [13], with men aged >45 being at greater risk of more severe manifestations. In terms of persistent diseases, the presence of an immunosuppression, a valvulopathy and vascular aneurysms were soon associated with evolution to endocarditis or vascular infections [13]. However, it was also hypothesized that clinical presentation could vary depending on the strain of *C. burnetii* involved. Initially, owing to the established nomenclature, a difference between strains causing 'acute Q fever' and strains causing 'chronic Q fever' was actively sought. A correlation between plasmid types and clinical features was observed [14]. Then, the availability of a genomic sequence enabled the development of efficient genotyping tools to describe the genetic diversity of *C. burnetii* strains [15], demonstrating that no systematic link existed between a given genotype and the acute or chronic form of the disease. For example, almost all genotypes could be represented in 'chronic' isolates of *C. burnetii* [15]. Thus, the evolution to a persistent form of Q fever appears to be largely dependent on host genetic factors, like the presence of an aortic bicuspid [16] or evolution to lymphoma after *C. burnetii* persistent infection [11].

Conversely, certain types of clinical expression of the primo infection or 'acute form' have been demonstrated to be dependent on the strain involved. For example, in French Guiana, the epidemiological profile of the disease is atypical [17,18]. Acute Q fever mainly consists of pneumonia characterized by a more severe initial presentation than community-acquired pneumonia caused by other microorganisms in this region [17,19]. Genotyping methods revealed that the epidemic strain causing Q fever in French Guiana was clonal and had only been reported in this territory [20,21]. This is a good illustration that clinical presentation of Q fever primo-infection and its epidemiology is dependent upon the strain involved [22].

The global advances provided by genomics helped to establish that the generic term of 'Q fever' covered a large diversity of clinical diseases and epidemiological profiles, depending largely upon the genetic properties of *C. burnetii* strains. We are beginning to understand this genetic diversity and its mechanisms. In this review we focus on what genomics has brought to all fields of the study of Q fever and detail the different stages of these advances.

• Pathophysiology/lifestyle

C. burnetii presents a very particular intracellular lifestyle. The bacterium has evolved to survive and multiply in the harshest of intracellular compartments: the phagolysosome [23]. The parasitophorous vacuole (PV) generated after *C. burnetii* entry its host cell progressively develops lysosomal characteristics with acidic pH, low oxygen tension, hydrolases and cationic peptides. Genomics has enabled an understanding of some of the mechanisms of this particular pathophysiology.

Secretion systems in *Legionellaceae* & *C. burnetii*

C. burnetii is closely related in evolutionary terms to *L. pneumophila* **(Figure 1)**. Bioinformatics approaches investigating the mechanism of infection have revealed a common virulence strategy for these bacteria [24]. Like *Legionella, C. burnetii* encodes a type IV secretion system (T4SS), allowing for the delivery of effector proteins into the host cytosol during infection [23]. Twenty-three of the 26 Dot/Icm T4SS proteins described in *L. pneumophila* were found to be homologous in *C. burnetii* [2].

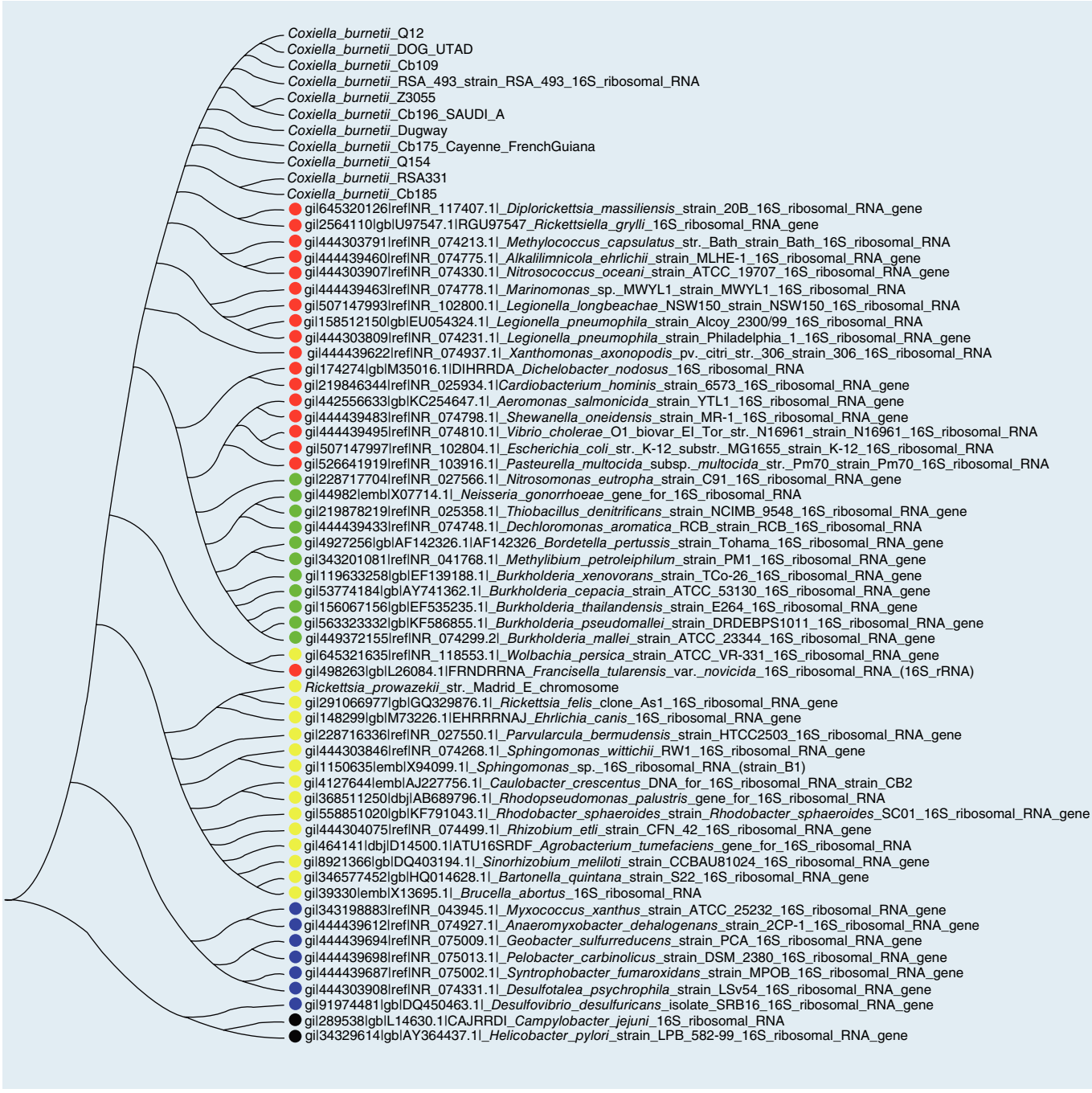

Figure 1. Phylogeny estimation of proteobacteria 16S rRNA genes. Evolutionary analyses were conducted using the MEGA6 tool. Multiple alignment was performed using the muscle method, and the tree was created with the NJ algorithm with 100 bootstrap iterations. α-proteobacteria are colored yellow, β-proteobacteria in green, δ-proteobacteria in blue, ε-proteobacteria in black and γ-proteobacteria in red. In the tree, we can observe *Coxiella burnetii* strains belonging to the γ-proteobacteria group.

Several studies have identified the substrates of *C. burnetii* Dot/Icm machinery. Initially, owing to the impossibility of obtaining *C. burnetii* mutants, the transformation of *L. pneumophila* was used. The candidate substrates of T4SS are characterized by eukaryotic-like domains able to mimic or inhibit host cellular processes [25,26]. Four *C. burnetii* Anks (AnkA, B, F and G) have been identified as the result of transformation of *L. pneumophila* [27]. The addition of AnkG to *L. pneumophila* enabled its replication in host cells by preventing

apoptosis, mimicking the way *C. burnetii* persists in its host cells [28]. This demonstrates that, although both *C. burnetii* and *L. pneumophila* virulence strategies are based on T4SS, they have some differences in effector proteins [28]. Furthermore, using *L. pneumophila* as a secretion model, 12 total plasmid effectors were found to be translocated into the host cell cytosol by the Dot/Icm T4SS during infection [29,30].

Recently, the development of axenic media has removed the constraints of genetic transformation of *C. burnetii* and allows transformants to be obtained in 16 days [5], instead of the 2–3 months previously required [31]. Thanks to the expression of recombinant proteins in *C. burnetii* using a shuttle plasmid, Chen *et al.* demonstrated the functionality of T4SS, confirming previous findings based on *L. pneumophila* transformation [32]. Moreover, *C. burnetii* mutants with deletion of genes *dotA* and *dotB*, which are components of type IVB secretion systems, appeared unable to secrete T4BSS substrates to infect the host cells and to produce the replication vacuole typical of *C. burnetii* [33]. Other mutants have been developed with transposon insertions in *IcmD* and *IcmL* genes that are essential components of the Dot/Icm T4SS [34–36]. These mutants are defective for intracellular replication. Conversely to what is observed in *L. pneumophila*, the Dot/Icm system of *C. burnetii* begins to be functional 8 h after the uptake by the host cell, after acidification and endocytic maturation of the *Coxiella*-containing vacuole [37].

Legionella and *Coxiella* also encode components of type 1 secretion system (T1SS). In *L. pneumophila*, it has been recently demonstrated that T1SS is functional and involved in the internalization of the bacterium into its host cells [38]. In *C. burnetii* Nine Mile, some effectors proteins of this system are mutated but T1SS system is possibly functional [21]. By contrast, in the Cb175 strain from French Guiana, T1SS operon is characterized by a large 6105 bp deletion and the same gene loss is observed in *L. longbeachae* [21]. Further experimental studies are needed to determine the exact role of T1SS in *C. burnetii* and to decipher the impact of such deletions in this region.

Response to stress conditions

Several studies have been performed to elucidate the *C. burnetii* protection strategy under the exceptionally stressful conditions of the phagolysosome. *C. burnetii* encodes an unusually high number of basic proteins, comparable to that found in *Helicobacter pylori*, which resides in the extremely acidic environment of the gastric mucosa [2]. These proteins are most probably involved in buffering the acidic environment of the phagolysosome. In the same manner, four predicted sodium ion/proton exchangers are present in *C. burnetii* genome. Furthermore, genes involved in vacuole detoxification and transporters for osmoprotectants have been identified, enabling the bacterium to face osmotic and oxidative stress conditions [2]. When it is outside of its cell host, *C. burnetii* is in a spore-like form that resists desiccation, chemical products, disinfectants and UV radiation [1]. It is also highly resistant to temperature stress, thus the stringency of pasteurization procedures was enhanced in the 1950s to eliminate *C. burnetii* [39]. A microarray transcriptional analysis was performed by Leroy *et al.* to study genes involved in the response to temperature stresses. Under cold and heat shock stress conditions, genes that are differentially expressed are organized into regulation clusters [40]. The same genes are differently expressed in these two different conditions. These genes are associated with membrane biogenesis, particularly lipopolysaccharide and peptidoglycan synthesis, bacterial division and bacterial sporulation [40].

Metabolism

C. burnetii's genome contains a high proportion of transporters for organic nutrients compared with *Chlamydia* and *Rickettsia*. A biosynthetic pathway is found for 11 amino acids, although none of them are complete. The presence of 15 amino acids and three peptide transporters suggests that the bacterium acquires these substrates from the host to compensate for this deficiency [2]. Enzymes for glucose metabolism, the electron transport chain, pentose phosphate pathway and tricarboxylic pathway are found. Pathways for lipids and phospholipids, purine and pyrimidine, coenzymes and cofactors are also intact. Pathways for the use of glucose, galactose, glycerol and xylose are present, in contrast to enzymes involved in cholesterol synthesis, with the exception of two sterol reductases (CBU1206 and CBU1158), suggesting the uptake and conversion of metabolites from the host, and not a *de novo* biosynthesis [2].

The genome

• Comparative genomics & the pan-genome study

Comparative genomics consists of studying the similarities and differences in genomes structures and functions between different microorganisms at the intra- or inter-specific level [41]. The first *C. burnetii* genome was sequenced in 2003; the Nine Mile Phase I RSA493 strain, isolated from a tick in 1935 [2]. It was composed of a 1,995,275 bp chromosome and a 37,393 bp QpH1 plasmid. This genome sequence enabled the *C. burnetii* genome to be compared with those of other intracellular bacteria. Several elements considerably differentiating *C. burnetii* from other sequenced intracellular organisms (*Rickettsia* and *Chlamydia*) and its closest related γ-proteobacteria (*Legionella*) have been described [2]. This genome was predicted to encode 2134 coding sequences, of which 719 were hypothetical with no equivalent in other γ-proteobacterial sequenced genomes. Moreover, a much higher percentage of the genome was coding, compared with other species known to have undergone genome reduction such as *Rickettsia prowazekii* or *Mycobacterium leprae*. Also, a high number of pseudogenes (83) were found, suggesting that genome reduction is still in progress. An unusually high number of 29 IS elements was reported [2], suggesting high genomic plasticity. Transportation, biosynthesis and metabolic capabilities were also greater than in other intracellular bacteria such as *Rickettsia* and *Chlamydia*. Finally, a new family of ankyrin repeat-containing proteins was described. The *C. burnetii* RSA493 strain also encoded some genes very similar to eukaryotic genes that did not match any prokaryotic genes sequences, reflecting its intracellular nature. As with *Legionella pneumophila, C. burnetii* encodes multiple eucaryotic-like proteins, probably to modulate its host cell functions. The genomic sequence of this first strain allowed for the specific features of *C. burnetii* to be described, with ongoing genome reduction probably due to a recent adaptation to intracellular parasitism [2].

Since this first genome publication, seven whole *C. burnetii* strain genome sequences have been published and are available in GenBank: Dugway 5J108–111 [42], CbuG_Q212 [2,42], CbuK_Q154 [42], RSA 331 [2,42], Nine Mile Phase I (NMI) [2,42], Z3055 [43] and Cb175_Cayenne_FrenchGuiana [21]. Sixteen incomplete genomes are also available: Cb109 [44], Q321, MSU Goat Q177, Cb_C2 [45], Cb_B1 [45], EV-Cb_BK10 [45], Cb_O184 [45], EV-Cb_C13 [45], Cb_B18 [45], Cb171_QLYMPHOMA, DOG UTAD [46], Cb196_Saudi_Arabia [47], Namibia [48], Cb185 [49], AuQ01 [50] and NL-Limburg. These new data enable a deeper comparative genomic analysis to be performed at an intraspecific level. To date, the largest *C. burnetii* genome reported is that of the Dugway strain, isolated from a rodent, with a 2,158,758 bp chromosome and a 54,179 bp QpDG plasmid. The smallest is that of Cb175, isolated from the heart valve of a patient from French Guiana, with a 1,989,565 bp chromosome and a 37,398 bp QpH1 plasmid [21]. All sequenced strains have an autonomously replicating plasmid (QpH1, QpDV, QpRS, QpDV) or plasmid sequences integrated into their chromosome [21]. The availability of whole genome sequences has enabled a pan-genomic analysis to be conducted on seven strains of *C. burnetii*. D'Amato *et al.* analyzed entire genomes to obtain an estimated percentage of core genes (genes present in all strains), accessory genes (genes present in some strains) and unique genes (present in only one strain), [21,43] using a blast score ratio algorithm [51,52]. The core genome/pan-genome ratio was 96%, with a total of 13,542 core genes, 498 accessory genes and 88 unique genes **(Figure 2)** [21]. Among the unique genes, 74 belonged to the Dugway strain, 13 to strain Q212 and one single gene to RSA331 [21]. This demonstrated a strong similarity between all considered *C. burnetii* strains and suggested that the pan-genome is closed; however, given the limited number of strains in the study, a more comprehensive analysis is needed to confirm this hypothesis. In addition, a detailed comparative analysis of some epidemic strains of *C. burnetii* has revealed some particularities, which are discussed below in the 'Pathogenicity & genomic characteristics of *C. burnetii* strains' section.

• Phylogenetic analysis

Initially, in the absence of genomic phylogenetic information and basing on ecologic similarities (e.g., culture methods), *C. burnetii* was assigned to the class α-proteobacteria, such as *Rickettsiales* and *Chlamydiales* [53]. Before whole genome sequencing, the sequencing of 16 rRNA excluded any relationship between *C. burnetii* and members of this class [54], rather showing a common line of descendent with *Legionella pneumophila* and *Rickettsiella grylli*, belonging to the

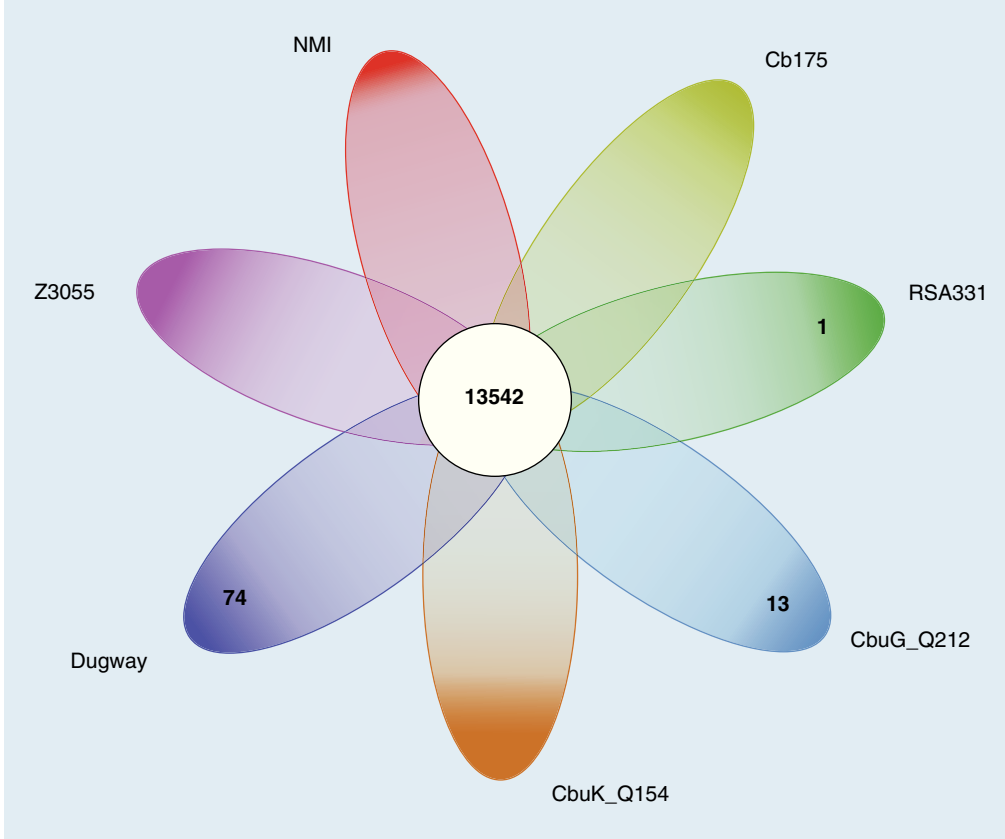

Figure 2. Pan-genome of *Coxiella burnetii*. Pan-genome representation: the middle of the flower represents the core genes, and the petals show the number of genes that are unique for each strain.

class γ-proteobacteria **(Figure 1)** [54,55]. Members of the *Legionella* genus can survive in the environment within free-living amoeba thanks to a mechanism of resistance to phagocytosis [56] and *C. burnetii* can also survive in a spore-like form in vacuoles of *Acanthamoeba castellani* [57].

Several studies have shown that horizontal gene transfers between intra-amoebal microorganisms take place in free-living amoebae [58–62]. On the genomic level, the presence of a high number of ankyrin repeat domains and a similar sterol δ-reductase gene in *Legionella* and *C. burnetii* genomes suggest that they exchange genes within the amoeba [58]. Similarly, some mycobacterial genes encoding for pyr-redox end up phylogenetically close to *C. burnetii* and other amoeba-resistant bacteria, suggesting abundant horizontal gene transfer in the amoeba [58]. In this context, the similarities of a genome sequence are a good illustration that *C. burnetii* shares a common ecosystem with other intra-amoebal pathogens.

For years, *C. burnetii* has been the only representative of its genus, preventing accurate phylogenetic studies about a putative ancestor. In 2000, *Coxiella cheraxi*, a new member of the genus showing 95.6% similarity with *C. burnetii* in 16S rRNA sequences, was isolated from crayfish in Australia [63]. However, the genome sequence of this bacterium is not available to date. More recently, several *Coxiella*-like organisms, isolated from non-vertebrate species (mainly ticks) were identified using 16SrRNA sequencing [64–67]. Some of these *Coxiella*-like organisms play the role of tick endosymbionts like *Coxiella*-like from the tick *Amblyomma americanum* (CLEAA) [64]. CLEAA shows a highly reduced genome (44) when compared with *C. burnetii*. Phylogenetic analysis based on rRNA 16S sequence and orthologous groups among *Coxiellaceae* and *Legionellaceae* have shown that CLEAA is closely related to *C. burnetii* but that it does not derive from *C. burnetii* [64]. Both species probably share a common ancestor. Recently, Gottlieb *et al.* [67] compared the genome of *Coxiella*-like symbiont of *Rhipicephalus turanicus* ticks (CRt) with other genomes of *Coxiella*

genus and *Legionellales*. It has a larger genome than CLEAA but a low protein-coding content (48.5%) and a high number of pseudogenes (675). Phylogenetic analysis also suggests a common ancestor between *C. burnetii*, CLEAA and CRt [67].

Duron *et al.* performed a phylogenetic study among *C. burnetii* and *Coxiella*-like tick endosymbionts, combining a new multilocus typing method and whole genome sequencing. This work revealed the presence of high diversity among *Coxiella*-like organisms, with four clades described (A–D) [65]. Interestingly, in this phylogenetic network, *C. burnetii* belonged to a unique subclade within the clade of *Coxiella* associated with soft ticks (clade A) **(Figure 3)**. This finding suggests that *C. burnetii* originated from a tick-borne *Coxiella* ancestor. There was also a wide distribution of *Coxiella*-like microorganism across tick species, and a large variety of genotypes, suggesting a long evolutionary history of the association between *Coxiella* and ticks. By contrast, the low genetic variability of *C. burnetii* strains suggests that a vertebrate pathogen recently emerged from a tick-associated ancestor [64,65].

These studies are major steps forward in the conception of *C. burnetii* phylogeny, recounting the story of an evolutionary transformation from a tick endosymbiont to a highly virulent

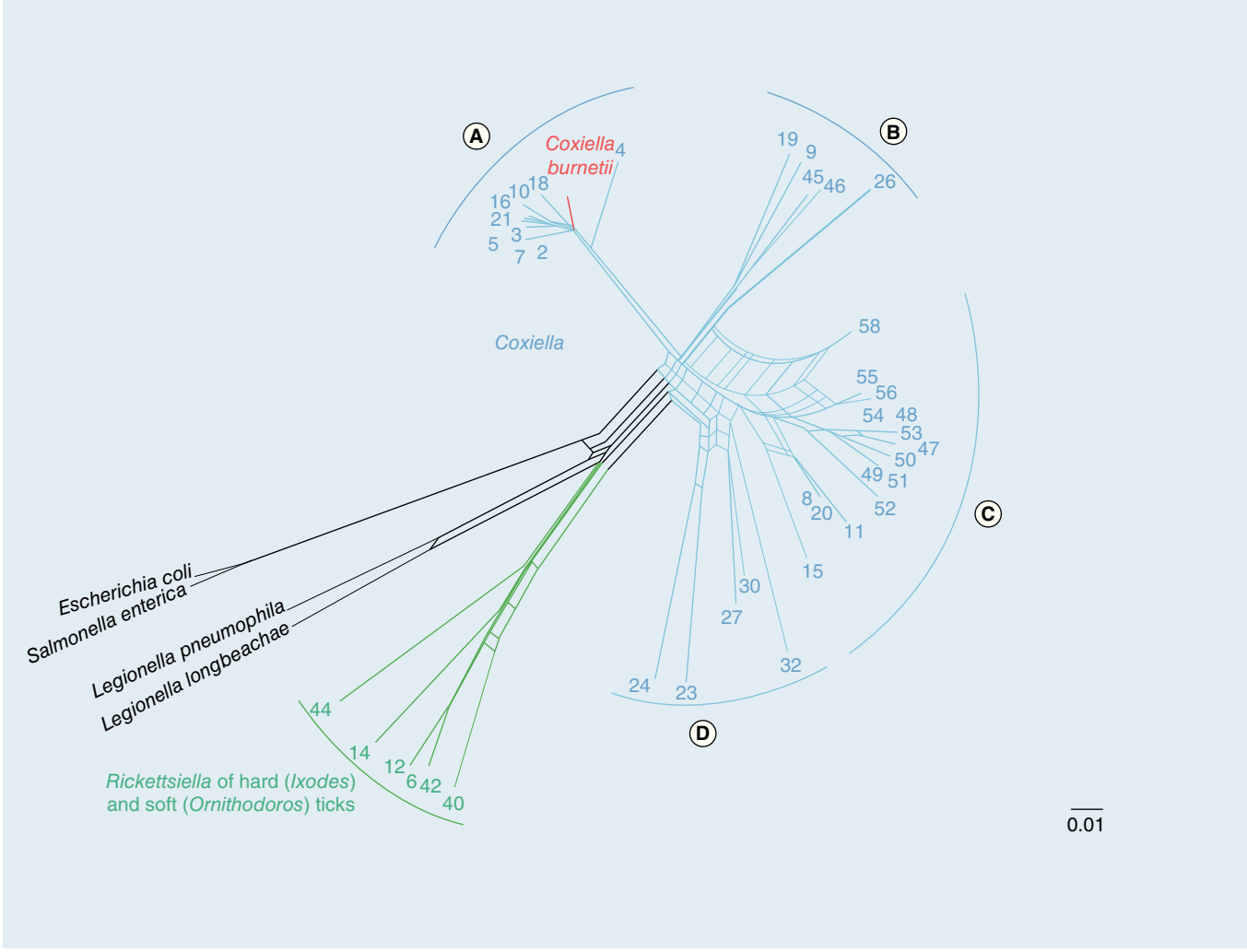

Figure 3. Phylogenetic network with concatenated 16S rRNA, 23S rRNA, GroEL, rpoB and dnaK sequences (3009 unambiguously aligned bp), including 71 *Coxiella*-like strains of ticks, 15 *C. burnetii* reference strains and bacterial outgroups. (A) *Coxiella* of soft ticks (*Argas*, *Ornithodoros*) and *Coxiella burnetii*; **(B)** *Coxiella* of hard (*Ixodes*, *Amblyomma*) and soft (*Omithodoros*) ticks; **(C)** *Coxiella* of hard (*Rhipicophalus*) and soft (*Ornithodoros*) ticks; **(D)** *Coxiella* of hard ticks (*Amblyomma*, *Dermacentor*, *Haemaphysalis*).
Reproduced with permission from [65].

vertebrate pathogen. By comparing the gene content of *Coxiella-like* endosymbionts with *C. burnetii* we observed a higher gene content in *C. burnetii*. This trend is also observed in *Buchnera*, an endosymbiont [68] of aphid species that is very close to pathogenic *E. coli* **(Box 1)** [68–72].

Genotyping

Several methodologies have been developed for differentiating *Coxiella burnetii* isolates according to their environment, hosts and pathogenicity. It was soon demonstrated that classical genotyping methods, such as 16S ribosomal RNA sequencing, 16S-23S ribosomal DNA (rDNA) sequencing, internal transcribed spacer sequencing and RNA polymerase's β-subunit (rpoB) sequencing were not relevant, having no sufficient discriminant power in *C. burnetii* [73–76]. Thus, many attempts were made to find a good differentiating method that could be used routinely. Before the availability of a genomic sequence, restriction fragment length polymorphism (RFLP) analysis of genomic DNA and PCR-RFLP [77–79] was used and did reveal genetic diversity, but these methods were shown to have limited sensitivity and reproducibility. Differentiation of strains based on sequences or PCR-RFLP analysis of several genes, such as *Com1*, *DjlA* (*MucZ*) and *icd*, was also proposed [80–82]. Subsequently, owing to the availability of a genome sequence, a more global method using comparative genomic hybridization using a whole-genome microarray was performed, allowing for polymorphisms in chromosomal and plasmid open reading frame (ORF) sequences to be detected and determining the genetic profile of different strains basing on the absence/presence of genes indicated as 'genomotypes' [22,83]. Some of these genomotypes have been associated with host specificity and virulence, but this fastidious method cannot be performed routinely in a clinical setting. Currently, two discriminant sequence-based genotype methods are most frequently used: multispacer sequence typing (MST) [15], and multiple locus variable number of tandem repeats (VNTR) analysis (MLVA) [84,85].

VNTR-MLVA genotyping

Svraka *et al.* searched for tandem repeats in the RSA 493 strain sequence. They identified seven VNTR sequences, which were amplified from 21 *C. burnetii* strains [84], resulting in five major clusters and nine MLVA types. Subsequently, Arricau-Bouvery *et al.* [85] identified 36 MLVA genotypes from 42 *C. burnetii* isolates and proposed to use two panels of markers to enhance the discriminatory power of this method. This method has been applied in recent epidemiological studies in Germany and Poland [86,87], and shows a good discriminant power but lacks interlaboratory reproducibility [85].

MST & the concept of 'geotyping'

MST genotyping was developed in 2005. Glazunova *et al.* investigated the variability of sequences of spacers located between two ORF, which were supposed to present less selection pressure than adjacent genes. The authors identified ten highly variable spacers that allow three monophyletic groups [15] and 30 different sequences types to be defined from 173 *C. burnetii* isolates. Among these monophyletic groups, the second group contained only strains presenting a QpH1plasmid. Conversely, in the

Box 1. *Coxiella burnetii* and *Coxiella* endosymbiont.

- We compared the gene content of *Coxiella* endosymbiont of *Amblyomma americanum* (NZ_CP007541) and *Coxiella burnetii* by using Proteinortho [69] at parameters of 50% identity and 70% coverage.
- In *Coxiella* endosymbiont, 523 genes (i.e., 94% of its total genes) are shared with *C. burnetii*, while 1319 genes of *C. burnetii* are absent. These 1319 belong mostly to COG categories of transcription (K), cell wall/membrane/envelope biogenesis (M), replication, recombination and repair (L) and defence mechanisms (V). These findings are consistent with what has been previously reported in the case of *Buchnera* endosymbiont, compared with its closely related nonsymbiotic species *E. coli* [70,71,72], which showed a limited capacity for DNA recombination and repair (indicating a vulnerability to DNA damage), a low number of transcriptional regulators of genes involved in the envelope biogenesis (suggesting a weak surface structure) and few genes encoding ABC transporters [68]. Moreover, no loss was described for *Buchnera* in genes encoding Sec proteins, which are conserved with *E. coli* and other closely related species [68]. In *Coxiella*-like endosymbiont, these proteins are also conserved and we found the genes encoding for subunit SecE, SecB, YidC, SecA, SecY, YajC, SecG, SecD, and SecF of a preprotein translocase involved in secretion systems of type II, IV and V.

first group only QpDV and QpRS were represented while the third group showed only QpH1 or plasmidless strains. This illustrates that plasmid types are correlated with genotypes. Most of the genotypes are detected in both animals and humans [15,88–90], while others show host specificity [91]. This genotyping method is highly discriminant and has been used in several other studies to characterize strains from all over the world so that databases are available allowing for easy interlaboratory comparison [15]. The MST genotyping of a high number of strains from different geographical areas has helped to reconstitute the epidemiology of *C. burnetii* from one region to another and identify epidemic clones. Currently, some MST are spread across the five continents [15,88–89,92], while others are very specific to one geographical area. This is the case of MST 17 in French Guiana (discussed later in the text) [20] and MST 51 [93], a novel genotype detected in Saudi Arabia (strain Cb196) [47]. Thus, given the geographical distribution of strains, this method can be considered as a 'geotyping' tool, as illustrated in **Figure 4**. For example, genotype MST33 was found to be the predominant clone causing the historically most significant outbreak in The Netherlands [88,94–95] and a phylogenetic analysis revealed that this clone had probably spread from Germany to The Netherlands via France. MST 20 is found in Europe and in the USA, suggesting an historical spread of the disease by infected animals or humans.

As suggested by **Figure 4**, this 'geotyping' is still incomplete and should be improved by further studies to provide a complete cartography of the genetic diversity of *C. burnetii*.

• Pathogenicity & genomic characteristics of *C. burnetii* strains

Q fever clinical presentation and its link with *C. burnetii* strains was investigated at a very early stage in Q fever studies [96]. In the absence of an available genomic sequence, initial studies on this issue were based on the comparison of plasmid types [14,25–26,73,77,97–99], in an attempt to find a link between a given plasmid and the 'acute' or 'chronic' forms of the disease. Animal models have also been used to compare strains pathogenicity **(Box 2 & 3)** [100–104] but extrapolation of these results to human beings seems tenuous to date. Globally, these methods have failed to precisely describe virulence determinants in *C. burnetii* and this failure illustrates that the traditional dichotomy between 'acute' and 'chronic' Q fever is without biological foundation.

Gene loss & strain virulence

The development of sequencing methods and massive comparative genomic analyses have revolutionized the paradigm of virulence in microbiology. During the pregenomic era, the virulence of bacteria was thought to be linked to the acquisition of 'virulence genes.' However, several studies have shown that some genes supposed to code for 'virulence factors' were also present in nonpathogenic species [105–108]. It has now been demonstrated that specialization of bacteria to eukaryotic hosts and evolution to hyper pathogenicity is rather driven by genome reduction with massive gene loss [105]. This is illustrated by a recent study comparing the 12 most dangerous bacterial species through recorded history for humans ('bad bugs') to their phylogenetically closest, but nonepidemic species. Ten functional COG categories contained significantly fewer genes in 'bad bugs' [109,110]. Another example of genomic reduction has been found in *Mycobacterium*. Leprosy bacillus lost 2000 genes after its divergence from the last common mycobacterial ancestor [111–116]. This phenomenon has also been observed between *Shigella* spp and *E. coli* [117,118].

Rickettsiales have represented the best model for studying reductive evolution [106,119]. Sequencing of the *Rickettsia prowazekii* genome, the most dangerous epidemic member of the genus *Rickettsia*, has not shown the presence of virulence factors [119]. Genome comparison of *R. prowazekii* with the less virulent *R. conorii* revealed that *R. prowazekii* is a subset of *R. conorii* [106]. Similarly, a comparison of *R. africae* with highly pathogenic *R. rickettsii* showed a loss of genes in *R. rickettsii* [120]. In general, pathogenic *Rickettsia* species lack what have been defined as 'pathogenicity islands' [107]. During the passage from extracellular to intracellular lifestyle, *Rickettsiaceae* lost 2135 genes and, in particular, they lost the entire mismatch excision repair and a large part of the recombinational repair machinery. *R. prowazekii* and *R. typhi* have lost the greatest number of ancestral genes.

Taken together, these findings favor the hypothesis that genome reduction, due to genetic isolation in a specific host, consists of the loss of genes mainly involved in metabolic regulation pathways, repair machinery and secretion

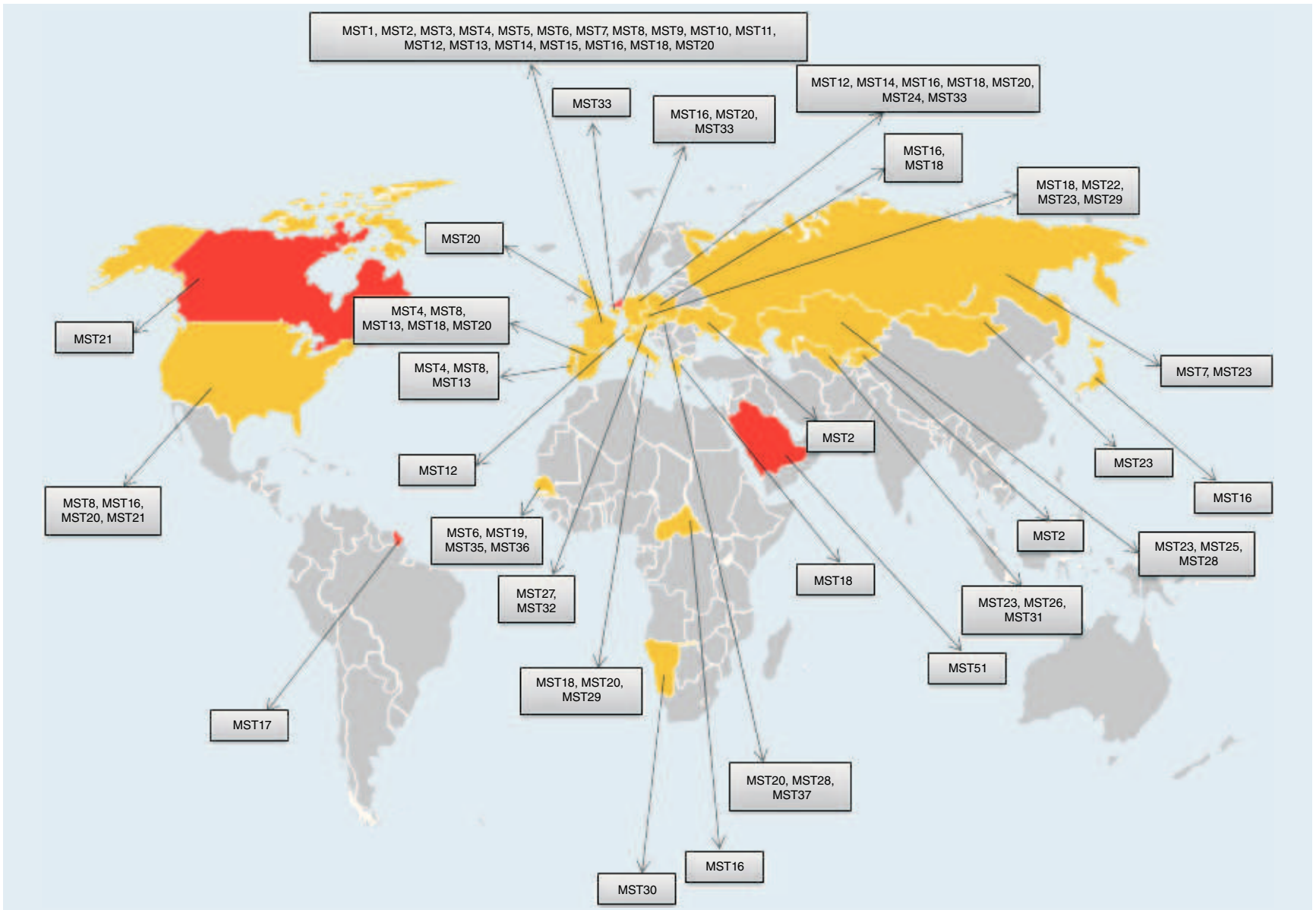

Figure 4. 'Geotyping.' Geographical distribution of *Coxiella burnetii* detected genotypes. In red, countries characterized by a unique circulating clone. In yellow, countries where other MST genotypes have been described.
MST: Multispacer sequence typing.

systems, leading to a deregulated multiplication in the host, which increases pathogenicity [109]. Thus, massive gene loss, combined with presence of TA and toxins (probably due to horizontal gene transfer) compose the virulent genomic repertoire of epidemic species. In the case of *C. burnetii*, genome sequencing of the hypervirulent strain from French Guiana [21] is a new example of links between virulence and gene loss.

French Guiana

Sporadic cases of Q fever have been described in French Guiana until 1998, when a study showed an increase in the seropositivity rate of Q fever from 1.9% in 1992 to 23.9% in 1996, in serum samples of febrile patients [121]. The annual incidence then increased from 37 cases per 100,000 inhabitants in 1996 to 150 cases per 100,000 inhabitants in 2005 [122]. A recent study confirmed that *Coxiella burnetii* was found in 24% of community-acquired pneumonia cases in French Guiana, the highest prevalence ever described in the literature [17]. The majority of cases diagnosed since 1996 occurred in Cayenne and its suburbs, indicating an unusual epidemiology of Q fever in this region [6], with no classical reservoir having been identified in previous environmental studies [6]. However, we recently detected *C. burnetii* by qPCR in stools, ticks and spleen samples of a dead three-toed sloth [123]. This animal is present in the rainforest hills surrounding Cayenne. We found a correlation between its reproductive season (the rainy season), and the number of cases of Q fever in Cayenne, with a time lag of between 1 and 2 months, suggesting that three-toed sloths could be the reservoir for the disease [124]. A clinical and serological comparison between 115 patients with Q fever from French Guiana and 182 from metropolitan France showed that acute Q fever has a higher incidence in Cayenne than in Marseilles, with pneumonia as the main clinical presentation and a uniquely strong serological response with high levels of Phase I IgG, suggesting the circulation of a hypervirulent clone [19]. After isolation of five *C. burnetii* from biological samples taken from patients from Cayenne (two with endocarditis and three with pneumonia), MST genotyping was performed and all samples showed a genotype MST17 [51]. This genotype seemed to be unique to French Guiana, which is very different from the situation in metropolitan France, where 21 different circulating genotypes have been identified [12]. Genome sequencing of the Cb175 strain, (isolated from a Guianese patient with endocarditis) has been performed [21]. Contrary to Z3055, Cb175 showed a significant difference compared with the reference Nine Mile strain: a large deletion consisting of 6105 bp, resulting in a genome reduction. This deletion has only been detected using a specific qPCR in eight other strains isolated from this territory and in none of 298 *C. burnetii* isolates from other areas, having different genotypes (8/8 vs 0/298; Fisher's exact test; $p < 0.0000001$) [21]. Genome reduction is consistent with previous findings that the most dangerous epidemic bacteria, compared with their closest nonepidemic species, are characterized by reduced genomes [112,120,125–129] and contain fewer secretion system proteins [109]. Interestingly, the missing region contained the T1SS hlyCABD operon and the same deletion is observed in *Legionella longbeachae*. Fuche *et al.* recently showed that T1SS is functional in *L. pneumophila* and is involved in the mechanism of internalization into host cells [130]. Further studies are needed to understand the exact mechanism by which this deletion in T1SS increases the virulence of MST 17 strains.

Canada

Multiple Q fever outbreaks have been recorded in Canada since 1982 [131] with parturient pets as the source of infection [132–134]; an outbreak involving three members of the same family exposed to a parturient dog was reported in 1994 in Nova Scotia [135]. We sequenced the strain responsible for this last outbreak; DOG UTAD that harbors the MST21 genotype, the only genotype detected in Canadian isolates to date [42,46]. The genome of this strain is very similar to Q212, another strain isolated from a Q fever endocarditis in Canada, harboring the same genotype. The DOG UTAD strain is plasmidless and shows 70 mutations in genes of which 47 are nonsynonymous compared with the Q212 strain. This low number of mutations suggests a very short genetic distance between these two strains, indicating a recent clonal radiation of MST21 in Canada [46].

Holland

The largest Q fever outbreak was reported in Netherlands between 2007 and 2010 [136]. The rapid intensification of goat farming contributed to the epidemics, with a serological attack rate of 92% [137]. A uniquely predominant genotype,

Box 2. Plasmid types and pathogenicity.

- First, a correlation of plasmid type with clinical issues was proposed [14]. Strains harboring QpH1 plasmid were associated with the acute form, while those harboring QpRS plasmid or QpRS chromosomal integrated sequence were associated with endocarditis, abortion and the other most 'chronic' forms of Q fever [77]. To investigate this correlation more thoroughly, it has been suggested that unique specific sequences for each plasmid were correlated with virulence. In this context, the gene cbbE', encoding for a surface protein, was found to be specific to plasmid QpRS [97] and the gene cbhE', encoding for a hydrophilic protein was specific to QpH1 plasmid [25]. However, some years later, the finding of a novel QpDV plasmid [98], detected in both 'acute' and 'chronic' isolates of *C. burnetii* suggested that the classification in plasmid groups was not sufficiently comprehensive [73]. Finally, Stein *et al*. demonstrated that the CbhE' gene specific to QpH1 plasmid could be detected by PCR in isolates from 'chronic' Q fever and was not systematically detected in isolates of 'acute' Q fever [73]. Further studies confirmed that neither type of plasmid nor gene unique to a type of plasmid could be used as reliable marker for distinguishing different pathotypes [26]. However, a recent correlation has been found between abortion in pregnant women and strains harboring a QpDV plasmid [99], although further analyses are needed to determine whether this result is correlated with specific plasmidic sequences or particular associated genomic virulence properties of these strains.

MST 33, was detected in dairy goatherds, in one sheep in the southern region of The Netherlands and on a farm in the eastern part of the country [138]. It represented 91% of identified genotypes, while nine other genotypes accounted for only 0.8% of all circulating genotypes. The most probable explanation is the emergence of a new genotype, responsible for abortion in dairy goats, which could spread more easily over the dense goat population in the southeast [138]. This genotype was found in samples from humans and from goats and sheep, confirming the hypothesis that goats and sheep are the source of the human Dutch Q fever outbreak [88]. MST33 probably spread to The Netherlands from Germany via France [88,95], because it was isolated in sporadic human cases in France in 1996, 1998 and 1999 and from the placenta of an asymptomatic ewe in Germany in 1992 [88].

Since the genome of The Netherlands outbreak strain is not available, a genomic analysis of strain Z3055 isolated from a ewe placenta in Germany in 1992 belonging to genotype MST33 was performed. A comparative genomic analysis using five other whole genomes available on GenBank at the time of the study was conducted [43]. Genome analysis showed an absence of gene loss or gain, with only slight differences to the reference Nine Mile consisting of point mutations, consistent with a clinical spectrum, which is not that different from that which has previously been described in the literature [139,140]. Interestingly, a high proportion of mutated proteins was found among membrane proteins, ankyrin repeat domain-containing proteins and proteins involved in transcription and translation processes. Similarly to the influenza virus [141], a possible explanation for the outbreak is that mutation in genes encoding for membrane proteins determined changes in surface antigens, allowing the clone to escape the host immune response of goats and spread rapidly [43].

• Diagnostic tools

Since *C. burnetii* is an intracellular bacterium with a fastidious culture, indirect diagnosis from clinical samples using serology (indirect immunofluorescence assay) has long been the gold standard for diagnosis in humans [142]. However, serology has the principal disadvantage of being positive after a significant delay of 1–2 weeks from the beginning of the infection [142]. Thanks to the analysis of the *C. burnetii* genome sequence, new tools have been developed for direct diagnosis. The identification of stable repetitive sequences has allowed efficient primers and probes for qPCR systems to be defined. The prediction of *C. burnetii* metabolic requirements from genomic sequence has accelerated the design of an axenic medium [4,5]. Furthermore, potential specific antigenic proteins can be deduced from the genomic sequence to try to develop more sensitive serological assays.

PCR & qPCR for direct diagnosis of Q fever

The first PCR systems detecting *C. burnetii* were developed before the availability of the entire genome sequence of the bacterium. Primers and probes for standard PCR systems were designed targeting sequences of different plasmid types [143], the superoxide dismutase gene, the *com1* gene, 16S-23S RNA and IS1111

repetitive elements, with detection limits ranging from 10 to 10^2 bacteria [75,144–147] in human and animal samples. Nested-PCR systems targeting IS111, *com1* and plasmid sequences have also been proposed [142,148–151].

RT-PCR or quantitative PCR (qPCR) has the advantages of both detecting and quantifying the bacteria from a given sample. Consequently, this method, which is also less time consuming, has superseded the standard PCR systems for routine diagnosis. Currently, the most sensitive RT-PCR system targets the IS1111 repetitive elements, present in approximately 20 copies in the *C. burnetii* genome [124,152–154]. This IS1111 qPCR allows for early detection of the bacterium in the serum of patients in the first 2 weeks of the infection, when serology is still negative, and in patients with Q fever endocarditis or vascular infections [155–158]. Another qPCR system targeting IS30A repetitive elements is also available, but is much less sensitive [124].

Genome-based design of culture medium

Recently, an axenic medium has been developed for *C. burnetii*, allowing host cell-free growth [4]. The first crucial step of the design of this medium has been to deduce metabolic pathway deficiencies from an *in silico* genomic analysis to design a medium that sustained a minimal metabolic activity for the bacterium [159]. Results suggested that in order to replicate in an axenic medium, the bacterium needed the presence of L-cysteine, which being a source of sulphur and a precursor in protein synthesis, provided a sufficient expression of ribosomal genes. Finding genes encoding cytochrome b, which has a high affinity for oxygen also explained the optimal replication in 2.5% oxygen [4]. In a second step, transcript profiles and phenotype microarrays were analyzed, leading to the formulation of the final medium known as ACCM2. Boden *et al.* recently achieved the first isolation of *C. burnetii* in ACCM2 from a clinical sample (heart valve). Thus, this medium has the potential to simplify the isolation of *C. burnetii* from clinical samples [160]. However, ACCM2 is mainly used for *C. burnetii* transformation in pure research and further studies are needed to test its efficiency for the isolation of *C. burnetii* from clinical samples and to develop its use as a diagnostic tool.

Serology: potential immunodominant antigens

The gold standard method for *C. burnetii* serology is immunofluorescence assay based on the serum antibody response to *C. burnetii* Nine Mile Phase I and Phase II. This technique requires culture and purification of the microorganism so that only specialized laboratories can carry out this method [142]. The development of new serological markers is an interesting challenge to improving the sensitivity and specificity of serological methods. In this context, the availability of a genome sequence has enabled the prediction of proteins produced by *C. burnetii* and their functions. This approach, combined

Box 3. Comparison of strain pathogenicity in animal models.

- The pathogenicity of several strains belonging to four different genomic groups (based on MST) has been compared in immunocompetent and immunocompromised mice and guinea pigs [100]. The authors found that development of the disease after infection with Priscilla strain and Q212 strain was slower and milder than infection with Nine Mile strain for example. Globally, strains belonging to genomic group I were responsible for more rapidly progressing and more severe disease and determined a stronger immune response than other strains [100,101]. Nine Mile strain appeared to be the most pathogenic, being the only one to cause an inflammatory reaction in infected mice [102]. However, these results should be interpreted with caution. Currently, comparing observations in experimental models with naturally infected humans could sometimes be erroneous in certain aspects and the research for a perfect animal model that truly mimics Q fever in humans is still a hurdle to overcome. *C. burnetii* is pathogenic but has low virulence in murine rodents, which show an inflammatory response with lower febrile disease and minimal signs of infection compared with humans. Large doses of Phase I *C. burnetii* are necessary to induce endocarditis in mice [100]. By contrast, in guinea pigs Q fever may represent a life-threatening disease [103]. It has been hypothesized that these differences are due to the ability of mice to restore affected cardiac valves [104]. For example, in the study by Samuel *et al.*, the Canadian strain Q212 [102] was much less pathogenic than Nine Mile Strain in a murine model. Nevertheless, this clone (MST 21) was the only one to be detected in Canada and to have triggered several outbreaks of both acute infections and endocarditis. Unfortunately, this is evidence that the human pathogenicity of a given *C. burnetii* strain cannot be deduced from an animal model.

with proteomic studies, has been applied to search for antigenic candidates for serodiagnosis of Q fever. Potential antigens belonging to COG categories of membrane proteins, translation and post-translational modification, DNA replication recombination and repair, proteins preventing DNA denaturation in stress conditions and heat shock proteins, have been selected [161]. Among these, OmpH, YbgF, Com1, OmpH, Mip, GroEL, DnaK, RplL, hsp60, Com-1, RecA, elongation factor Tu, OmpA-like transmembrane domain and FtsZ have been tested as potential Q fever markers [162]. However, these markers showed a moderate specificity when testing the microarray with convalescent sera from patients infected by *Rickettsia*, *Legionella* or other species close to *C. burnetii* [161–164]. Some authors have also tried to identify serological markers which are specific for a given clinical form of the disease. An immunodominant polypeptide of 28 Kilodaltons, the outer membrane protein adaA (acute disease antigen A) has been identified and proposed as a marker of acute Q fever [165]. It has been also detected as a potential specific marker for Q fever abortion in goats [166]. Differential protein expression involved in LPS biosynthesis between Phase I and Phase II of Nine Mile strain has also been studied [167]. However, to date, all these data need to be confirmed by further investigations to be applied in routine practice.

• Therapeutic tools

Antibiotic susceptibility

Currently, the treatment for Q fever is based on doxycycline, which is able to inhibit bacterial protein synthesis by targeting the 30S ribosomal subunit [1]. It has also been demonstrated that new generation antibiotics, such as telithromycin [168] and tigecycline, are effective against *C. burnetii* [169].

To date, some isolates have been shown to be resistant to doxycycline, such as the German strain Cb109 [44]. It was isolated from the cardiac valve of a patient with endocarditis who died during treatment. Analysis of the draft genome of this strain did not reveal any genetic differences with other strains that could explain its resistance; more detailed analysis could be useful to speculate on the reason for this important feature [44].

Thanks to genomics, some mechanisms of antibiotic resistance have been studied. For instance, genomics allowed for speculation on the mechanism of a high level of resistance to quinolones, which consisted of mutations in DNA gyrase (gyrA) genes resulting in amino acid substitution of Gly in place of Glu at position 87 and causing alterations to the GyrA protein, necessary for replication [170]. The fluoroquinolone-resistant *C. burnetii* Nine Mile I developed *in vitro* showed the upregulation of glutathione *S*-transferase and fabZ. The susceptible strain was characterized by upregulation of 13 proteins, of which one was an enhanced entry protein, reducing the ability to penetrate the host cell in the presence of quinolones [171]. The *C. burnetii* proteome has also been studied to investigate the mechanism of resistance to tetracycline and some proteins were found overexpressed in these conditions. Among these proteins there are: a signal peptidase I (involved in cell growth and division), a transaldolase (that provides NADPH cells responding to a higher energy requirement to excrete the antibiotic), the DNA polymerase III α subunit (which may induce resistance to antibiotics by mutagenicity) and the 3-methyl-2-oxobutanoate hydroxymethyltransferase (which reduces affinity between antibiotics and ribosome). Moreover, genes encoding the iron transport protein B, reducing activation of cytokine and factor pY, increasing synthesis of proteins to respond to protein synthesis inhibition determined by tetracycline are downregulated [172].

Putative antigens: surfaceome & vaccinology

Since Q fever in humans is often an occupational hazard, vaccination is considered in exposed populations and immunocompromised patients, or those with cardiac valvular dysfunctions [1]. To date, three types of vaccine have been proposed for Q fever: the attenuated live vaccine, produced and tested in Russia but discarded because of possible consequences for human health; the chloroform-methanol residue extracted vaccine or other extracted vaccines, which have been tested in animals but not in humans; and the whole-cell formalin-inactivated vaccine (Q-Vax), considered to be quite safe for humans and with 98% efficacy [173]. Zhang *et al.* combined 2-D gel and genomics to study possible specific proteins of *C. burnetii* that could be used as an antigen for the development of vaccines. After screening a genomic DNA library with convalescent-phase sera from mice, 20 different immunoreactive proteins

were cloned and identified [81]. Further studies are needed to confirm the possible use of these proteins as vaccine candidates.

Conclusion

The introduction of genomics has led to important changes in the management of Q fever and the study of *Coxiella burnetii*. It has been possible to speculate on genetic diversity and evolution by performing comparative genomic analysis and accurate phylogeny. The epidemiology of Q fever around the world is better known thanks to the description of 'geotypes.' Genomics also enabled the development of new diagnostic tools, based on qPCR, genotyping of strains and axenic culture. The detection of putative mechanisms of resistance to antibiotics, as well as the immunodominant antigens, which are potential targets for vaccines are also important developing fields.

In particular, the sequencing and analysis of the *C. burnetii* genome of epidemic strains made it possible to study the potential links between pathogenicity and genetic properties. The discovery of a unique genome reduction in the hypervirulent strain from French Guiana is the best illustration of this phenomenon. This is a crucial breakthrough for studying Q fever epidemiology, showing that *C. burnetii* causes more or less severe clinical presentation during primo infection, depending mainly on genomic determinants. On the contrary, no genetic determinants appear to be linked to a 'chronic' or 'acute' form of the disease. The persistence of the disease in an infectious focus seems to be largely determined by predisposing host factors (age, sex, valvulopathy, vascular aneurysms, joint prosthesis and pregnancy).

We are only at the beginning of the description of *C. burnetii* genetic diversity and its links with pathogenicity and epidemiology.

Future perspective

A more comprehensive pan-genome analysis including more than 40 strains collected in our laboratory is currently underway to provide an overview of *C. burnetii* clonal diversity and its relation to virulence. These advances will significantly help clinicians and epidemiologists with the diagnosis and management of Q fever around the world.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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EXECUTIVE SUMMARY

- Genomics is crucial for the rapid progress in the knowledge of *Coxiella burnetii* and to improve the management of Q fever.
- Genomic phylogenetic information allowed understanding the correct taxonomy of *C. burnetii* that belongs to the class of γ-proteobacteria.
- By using multispacer sequence typing that can be considered a 'geotyping' tool, we obtained a complete cartography of the genetic diversity of *C. burnetii*.
- Genomics helped to develop the acidified cystein citrate medium axenic medium that allows host cell-free growth of *C. burnetii* and enabled the genetic manipulation of this bacterium.
- We discussed the dichotomy between 'acute' and 'chronic' Q fever. This is without biological foundation; the genomic determinants of each strain are mainly responsible for more or less severe clinical presentation during primo infection.
- Genome sequencing of strains from exceptional epidemic situations has enabled the description of links between strains' genetic profile and virulence.
- In the case of the Cb175 strain isolated in French Guiana, this implicates gene loss and genomic reduction of more than 6 kb.
- In the case of strain Z3055 linked to The Netherlands outbreak, mutation of surface antigens helped the clone to escape host immune response and to spread among population determining the epidemics.
- A preliminary pan-genomic study using seven whole available genomes, suggested that *C. burnetii* has a closed pangenome.

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Article 4 : Loss of TSS1 in hypervirulent *Coxiella burnetii* 175, the causative agent of Q fever in French Guiana

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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

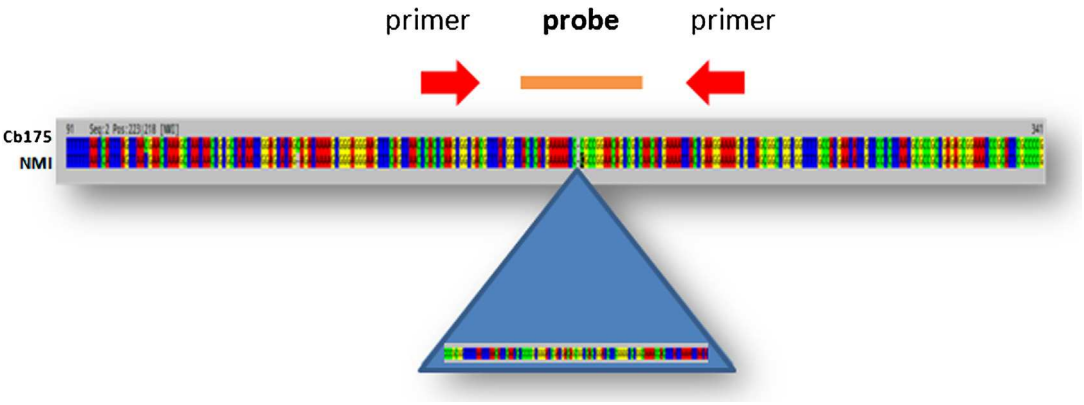

Fig. 1. The qPCR system used in this study. In orange, the probe targeting 23 bp of the Cb175 sequence containing the missing region. In red, primers flanking that region (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

match) to 0.0 (no significant alignment). Lost genes were found to have a blast score ratio ≤0.4 (having an identity ≤40% over 100% of the peptide length). A hierarchical clustering of strains based on genetic differences was obtained using the multi-experiment viewer (MeV) [24,25] by entering the value of the blast score ratio across groups. In this way, the Blast Score Ratio algorithm also allowed us to obtain an estimate of the genetic repertoire of the group of *C. burnetii* strains in our study, as previously described [17]. Therefore, genes with a blast score ratio >0.4 in some genomes, but not in all (i.e., genes that were found in some *C. burnetii* strains) have been classified as accessory, genes having a blast score ratio >0.4 in only one strain, but ≤0.4 in others were classified as unique, and genes having a blast score ratio >0.4 in all strains were classified as unique core genes. To obtain an estimate of the core genome/pangenome ratio, we considered: for the pangenome, the total number of genes before dereplication; for the core genome, the total of the genes of the core obtained by blast score ratio and genes from the filtered dataset of dereplication and not belonging to accessory or unique genes in multiple copies found in the same dataset.

The Cb175 and the reference NMI genomes were compared using the Probabilistic Variant Detection tool of the CLC Genomics Workbench 7.0 (Copyright CLC bio) with the parameters of length fraction = 0.7 and similarity fraction = 0.8 to identify Single- and Multiple-Nucleotide Variations (SNVs and MNVs), Insertions and Deletions. We confirmed or excluded larger InDels, when detected in the consensus sequence, by molecular biology during the finishing step.

Only mutations with coverage > 50 were considered. Protein sequences were aligned to verify whether point mutations were silent mutations or changed the amino acid sequence (non-synonymous mutations).

Genes of NMI and non-synonymous genes were classified by Clusters of Orthologous Groups (COG) using the WebMGA annotation tool [26]. Then, the proportion of different proteins for each COG category was calculated.

3. Results

3.1. General features

Cb175 is characterized by a 1,989,565 Mb chromosome (G + C content 42.6%) and a QpH1 plasmid of 37.388 bp (accession number HG825990 for both chromosome and plasmid). Based on PCR results, the Cb175 genome showed 100% identity and coverage with the sequences of Cox2.3, Cox5.8, Cox18.5, Cox20.7, Cox22.4, Cox37.1, Cox51.10, Cox56.4, Cox56.8, Cox57.6, and Cox61.2, suggesting that the genotype of Cb175, MST17, is similar to the other strains isolated in Guiana [7,11].

3.2. Blast score ratio and genetic variability

Genomic clustering using the blast score ratio algorithm demonstrated a high level of similarity between the strains, and also showed that Cb175 is in the cluster that includes strains NMI, RSA331 and Z3055, which harbor the plasmid QpH1 (Fig. 2). Based on the Blast score ratio algorithm, we estimated a core genome/pangenome ratio of 96%. Indeed, a total of 13,542 core genes (including 2270 unique core genes among 2568 total dereplicated genes), 498 accessory genes (including 210 unique genes)

Table 1
Panel of 15 *C. burnetii* strains used for RT-PCR.

Strain name	Nature of specimens	Clinical characteristic	Age/sex	Geographical area	Genotype	Ref.
Cb 179	Blood	Acute Q fever	44/M	Cayenne	MST17	[11]
Cb 181	Blood	Acute Q fever	55/F	Cayenne	MST17	[11]
Cb 182	Blood	Acute Q fever	47/M	Cayenne	MST17	[11]
Cb 77	Cardiac valve	Q fever endocarditis	40/M	Cayenne	MST17	[11]
Cb 176	Cardiac valve	Q fever Vascular infection	66/M	Cayenne	MST17	
Cb196	Cardiac valve	Q fever endocarditis	13/M	Saudi Arabia	MST51	[40]
Cb195	Cardiac valve	Q fever endocarditis	13/M	United states	MST54	
Cb165	Cardiac valve	Q fever endocarditis	75/M	France	MST1	
Cb173	Cardiac valve	Q fever endocarditis	66/M	France	MST8	
Cb163	Blood	Q fever endocarditis	12/F	Marseille, France	MST33	
Cb185	Placenta	Abortion	20/F	France	MST18	
Cb177	Cardiac valve	Q fever endocarditis	40/M	France	MST12	
Cb119	Cardiac valve	Q fever endocarditis	44/M	Senegal	MST19	
Dog Utad	Uterus			Canada	MST21	
Nine Mile	Tick			United states	MST16	

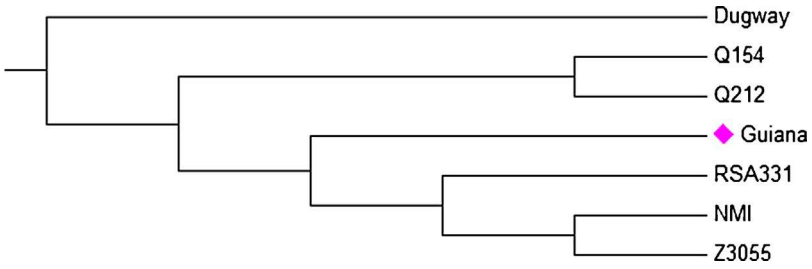

Fig. 2. Hierarchical clustering based on the blast score ratio across 7 whole analyzed genomes. The Guiana strain is in the same cluster of the other strains harboring plasmid QpH1, RSA331, Z3055 and NMI.

and 88 unique genes (not in multiple copies), were identified in these strains. Among the unique genes, 74 belonged to strain Dugway, 13 to strain Q212 and 1 unique gene to RSA331, as previously described [17]. Reads resulting unmapped against the Nine Mile I chromosome (NC_002971.3) have been assembled using CLC Genomics Workbench 7.0 with the parameters of minimum contig length = 85, length fraction = 0.5 and similarity fraction = 0.8. Then, the obtained contigs were aligned using BLASTN against the nr database and after only against plasmids' sequences database. Among 131 total contigs, 51 contigs showed a match with *C. burnetii* and all of these corresponded to a plasmid sequence, with more hits for the QpH1 plasmid.

The others remaining assembled contigs corresponded to *Mus musculus*, *Plasmodium yoelii yoelii*, *Francisella tularensis*, *Trypanosoma congolens*, *Staphylococcus epidermidis*, *Cricetulus griseus*, *Bacillus sp.*, *Staphylococcus epidermidis*, and *Influenza A virus*, representing the contaminants in our sample. No contig has been found to have a match with a bacterium close to *Coxiella* species. Therefore, no evidence suggested the presence of a unique gene specific to strain Cb175. However, we must consider that the small size of reads could represent a limit for assembly. This is, by the way, the reason for our choice to perform mapping to obtain chromosome and plasmid sequences rather than an assembly.

3.3. Point mutation analysis

A total of 1163 mutations between Cb175 and NMI were identified, among which 401 were located in intergenic regions and 762 were in coding regions. Among the 762 mutations in coding genes, 730 were SNPs (including 463 non-synonymous), 9 were insertion mutations and 23 were deletions (Supplementary Table 1). These mutated genes correspond to 397 non-synonymous genes. Mutated genes encoded proteins that are involved in the following processes: 137, metabolism; 26, replication; 4, transcription; 13, translation; 5, secretion system; 1, the two component system; 18, cellular processes, 3, defense mechanisms and 47 are membrane proteins, 9 are transporters, 4 are ankyrins, 1 is a chaperonin, 1 is a CRISPR and 128 are hypothetical proteins. Statistical analyses suggested a trend for an increase in the proportion of mutated genes that encode ankyrin repeat domain proteins compared with other proteins (4/9 vs. 393/1904, bilateral Mid-P test $p = 0.12$).

When proteins were classified by COG categories, the proportion of mutated proteins was significantly increased for category M (cell wall/membrane/envelope biogenesis) compared with the other categories (31/108 vs. 366/1805; bilateral chi^2 test $p = 0.04$). There was a trend toward an increase in the proportion of mutated proteins in category E (amino acid transport and metabolism, 23/86 vs. 374/1827; $p = 0.16$) and category H (coenzyme transport and metabolism, 21/77 vs. 376/1836; $p = 0.15$).

3.4. Missing region compared with the NMI' genome

PCR amplification of a gap to close with an expected size of 6869 bp yielded a band of 767 bp. Sequencing the 767 bp band followed by a BLASTN analysis [14] against the reference strain Nine Mile I showed that the sequence matched at 546 bp and 224 bp, flanking a missing region of 6105 bp in strain Cb175 (Fig. 3). The deleted region contains one pseudogene (CBU_2034) that corresponds to a hypothetical membrane-associated protein, one gene (CBU_2036) encoding a hypothetical protein, one pseudogene (CBU_2037) corresponding to a multidrug resistance ABC transporter ATP-binding and permease, one pseudogene (CBU_2038a) corresponding to a α-hemolysin translocation ATP-binding protein HlyB (*Escherichia coli*), and one gene (CBU_2040) encoding a HlyD family type I secretion membrane fusion protein. HlyA and HlyC, which are two important components of the *E. coli* Type 1 Secretion System (T1SS) hlyCABD operon, are not present in *C. burnetii*.

In all samples isolated from patients and ticks from French Guiana, the 6869 bp region was not amplified, but a 767 bp fragment similar to that of strain Cb175 was obtained. These results were corroborated with the qPCR analysis that was specifically designed to target the deleted region. Only DNA samples from patient and ticks from Cayenne tested positive in this qPCR assay, while those of all other genotypes tested negative, suggesting that the qPCR assay was specific for the MST 17 genotype.

3.5. No detection of the deletion in other sequenced strains and genomes available in GenBank

The deletion described earlier was not found in any other sequenced strains. The average coverage results of mapping and consensus size indicated that the 6105 bp region is present in all other C. *burnetii* genomes analyzed. Specifically, for strains sequenced using the SOLiD and 454 methods, we found an average coverage ranging from a minimum of 61 bp (for Cb121) to a maximum of 247 bp (for Cb13) with a consensus size of at least 5998 bp. For strains sequenced by Illumina, the minimum average value of coverage that we obtained was 54 bp (for Cb94) and the maximum value was 226 bp (as for Cb111) with a consensus size of at least 6089 bp. Moreover, the strains available in GenBank were not characterized by the loss of this region; each genome matched with a sequence identity value of 99–100%.

3.6. Alignment of the missing region against Legionella pneumophila genomes

Alignment of the 6105 bp sequence against *Legionella pneumophila* genomes revealed the presence of conserved regions in the lssB and HlyD genes. Specifically, in *Legionella pneumophila philadelphia1*, the following two genes were in the same region and situated next to each other: LPE509_01686

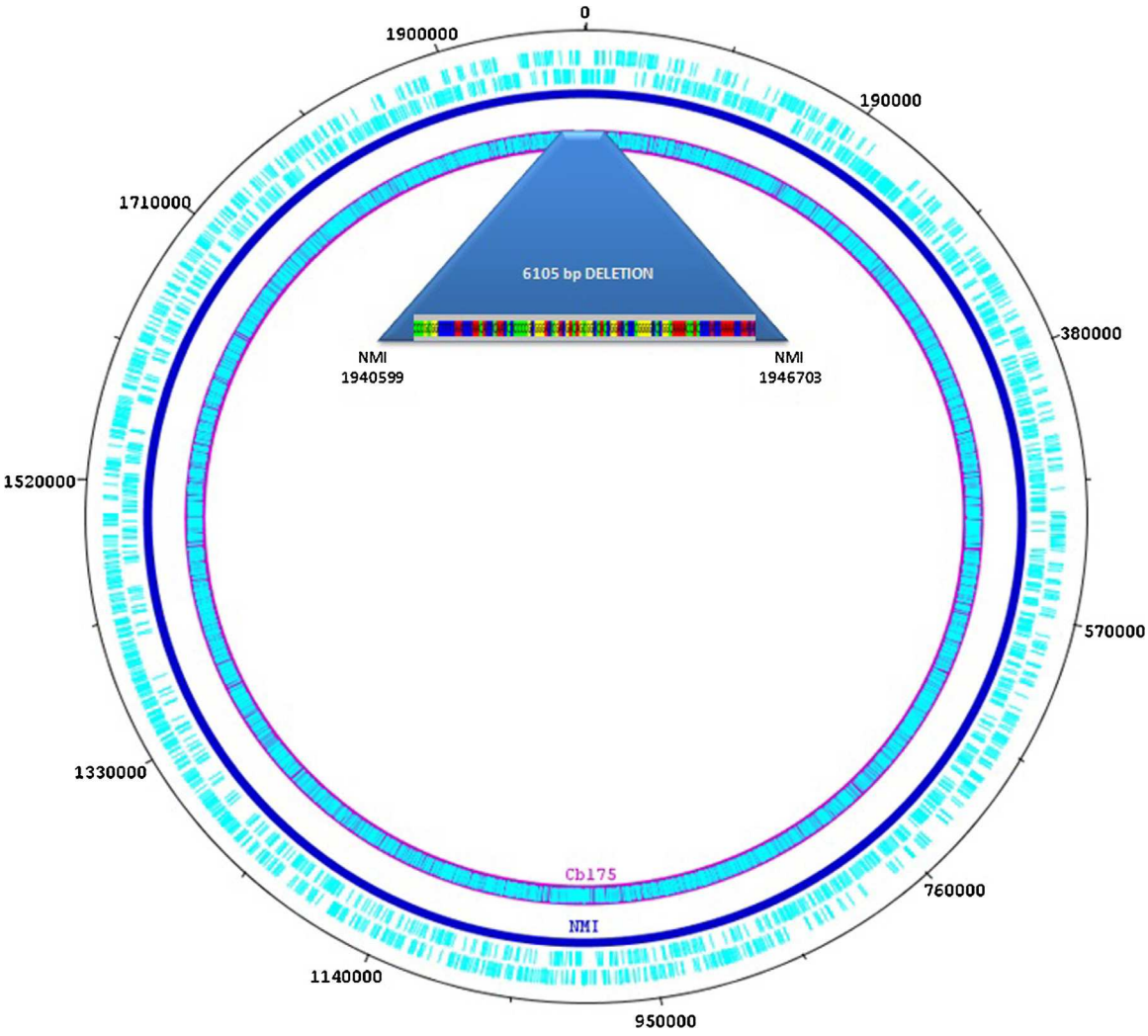

Fig. 3. Circular genomic comparison between Cb175 and NMI. In blue, NMI genome. In fuchsia, the Cb175'genome. On the Cb175 chromosome, the missing region is marked (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

HlyD family secretion protein (strain: Philadelphia 1, sub-species: pneumophila), sequence: NC_020521.1 (1791946.1793082, complement) and LPE509_01687 Legionella secretion system protein B (lssB) (strain: Philadelphia 1, sub-species: pneumophila), sequence: NC_020521.1 (1793086.1795071, complement). LssB (ABC-transporter-ATP binding) and lssD (HlyD family secretion protein) were also missing from the *Legionella longbeachae* compared with the *Legionella pneumophila* genomes [27,28].

4. Discussion

A comparative genomic analysis of strain Cb175, based on the Blast score ratio algorithm with 6 other available complete genomes of *C. burnetii* strains, showed low variability. Additionally, although the number of strains was relatively small and considering that the estimated ratio core genome/pangenome is high (96%), this study suggests that the pangenome of *C. burnetii* is closed [27].

C. burnetii 175 is an epidemic strain that causes a higher prevalence of acute Q fever than almost all other known genotypes of *C. burnetii* [7,10]. Genome analysis demonstrated a total of 397 mutated genes, including 137 that encode metabolic proteins and a trend for an increasing proportion of non-synonymous mutations in genes involved in ankyrin repeat domain proteins, amino acid metabolism, coenzyme transport and membrane or envelope biogenesis. [29]. A similar trend has been observed for strain Z3055 that is genotypically related to the epidemic strain from the outbreak in the Netherlands [17]. However, it is more likely that a big deletion has had phenotypic effects than these mutations.

Additionally, the size of the Cb175 genome (1,989,565 bp) was reduced compared with that of the Nine Mile I strain (1,995,275 bp). During the pregenomic era, the understanding of bacterial virulence was based on studies demonstrating that removal of certain genes from pathogenic species eliminated their capacity to infect hosts. Biased by an anthropocentric perspective, the term "virulence factors" was coined to describe such genes [30]. However in the comparative genomics era, highly pathogenic bacteria, such as *Mycobacteria* and *Rickettsia*, have been shown to have reduced genome sizes [31–36]. Subsequently, it has been demonstrated that the evolution to hyper pathogenicity can be driven by genome reduction, resulting in the inactivation or deletion of non-virulence genes [37]. A recent study that compared the 12 most dangerous epidemic bacteria with their closest non-epidemic species found that epidemic species are characterized by reduced genomes and are accompanied by a significant reduction in ORF content and a gradual disappearance of genes [29].

By performing PCR and a BLASTN search against the reference *C. burnetii* strain Nine Mile I, we discovered that a 6105 bp region was missing in strain Cb175. To date, this deletion is only and specifically detected in *C. burnetii* strains from French Guiana (8/8 vs 0/298, Fisher's exact test, $p < 0.0000001$). This deleted region

contains proteins involved in the T1SS. Protein secretion systems play roles as communication ports with eukaryotic cells and are therefore considered to be an integral part of the bacterial virulence arsenal. However, it was recently shown that some of the most dangerous epidemic bacterial species contain significantly fewer secretion system proteins than their closest non-epidemic relatives [29].

The same genes have also been shown to be lost in *L. longbeachae* [27,28]. *Coxiella* is a genus that is closely related to *Legionella* [38]. Therefore, we compared the missing region of *C. burnetii* 175 with that of *L. pneumophila*, the primary human pathogenic bacterium of the genus [39]. In *L. pneumophila*, T1SS genes are located in the same region and T1SS is functional, playing a role in internalization into its host cell [39]. In *C. burnetii* Nine Mile, the HlyB protein was mutated and two pseudogenes remained: HlyA is absent and HlyD is still complete and possibly functional. However, the influence of the deletion that we found on the functionality in *C. burnetii* 175 cannot be determined in the absence of experimental studies on this subject. For *L. longbeachae*, both hypotheses of an alternative T1SS system (using a different secretion machinery) and of a non-functional T1SS can be found in the literature [28]. In our work concerning the highly virulent *C. burnetii* 175 strain, the only observable effect of this deletion is a genome reduction (in contrast to *L. longbeachae*), which is comparable to what can be found in most virulent and epidemic microorganisms.

5. Conclusion

C. burnetii 175, a representative strain of the agent of Q fever in French Guiana, causing 24% of community acquired pneumonia, exhibits genome reduction due to a deletion in T1SS, which is specific to guianan genotype 17 as showed by qPCR results. Indeed, missing region was only detected in 8 other *C. burnetii* strains isolated from this territory and is absent from the 298 other strains that we tested in qPCR. This genome reduction is consistent with previous findings that showed that virulent or epidemic strains do not exhibit more genes or more virulence factors, but instead can be characterized by progressive gene loss. The particularly high prevalence and clinical severity of acute Q fever in French Guiana may be linked to these genomic features.

Competing interests and funding

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cimid.2015.04.003

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Article 5 : Antibiotic susceptibility determination for six strains of *Coxiella burnetii* MST 17 from Cayenne, French Guiana.

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Letter to the Editor

Antibiotic susceptibility determination for six strains of *Coxiella burnetii* MST 17 from Cayenne, French Guiana

Sir,

Coxiella burnetii is the causative agent of Q fever, a zoonotic infectious disease that is a public health problem in French Guiana [1]. Q fever represents 24% of hospitalised community-acquired pneumonia in French Guiana and is characterised by a severe initial presentation and an atypical serological response with high levels of phase I IgG despite clinical acute forms of the disease. We isolated six strains of *C. burnetii* from patients in Cayenne, the capital city of French Guiana [1]. These strains all belonged to a unique genotype, multispacer sequence type (MST) 17, which to date have only been isolated from this territory [1]. The particularly severe forms of Q fever in this region appear to be related to this genotype, which shows a large deletion in the type 1 secretion system (T1SS), resulting in genome reduction [2]. Because of the exceptional incidence of Q fever pneumonia in Cayenne, empirical antibiotherapy for community-acquired pneumonia in Cayenne hospitals comprises doxycycline and amoxicillin.

Flow cytometry has been used as a method for determining antimicrobial susceptibility for various micro-organisms using different properties of this technique (quantification, membrane characteristics, viability marker) [3]. For bacteria such as *C. burnetii* that are cultivated on cells, flow cytometry has the advantage of detecting bacteria separately from their host cells owing to differential properties of size and structural densities and the possibility to use specific antibodies coupled with fluorescent probes. We performed antibiotic susceptibility determination for six strains of *C. burnetii* genotype 17 isolated from patients from French Guiana using flow cytometry.

The six isolates of *C. burnetii* from French Guiana were cultivated on HEL cells in shell vials. Anti-*C. burnetii* rabbit antibody was added and incubated for 30 min at 37 °C and then goat anti-rabbit globulin coupled to fluorescein (Jackson ImmunoResearch) was added. Each sample was then analysed using a BD Accuri™ C6 Flow Cytometer (BD Biosciences). Operating conditions included log scales on laser detectors [forward scatter (FSC), side scatter (SSC) and fluorescence detectors (FL1)]. Each sample was analysed for at least 5000 events. The cytometric protocol was set for the size and complexity of the analysed particles as well as the intensity of the fluorescent probes used. Gating was performed on differentiate HEL cells from *C. burnetii* using SSC and FL1 (Fig. 1) to assess *C. burnetii* cell concentrations at Days 0 and 15. Growth was measured by comparing the number of *C. burnetii* copies detected by flow cytometry at Days 0 and 15. The growth ratio for each strain was calculated as log(number of *C. burnetii* copies at Day 15)/log(number of *C. burnetii* copies at Day 0). Each experiment was carried out in duplicate. Doxycycline, minocycline, levofloxacin, sulfamethoxazole/trimethoprim (400/80 mg, respectively), rifampicin, erythromycin, tigecycline, telithromycin and azithromycin were used. For each antibiotic, concentrations from 0.06 mg/L to 8 mg/L were tested, except for sulfamethoxazole/trimethoprim (8–64 mg/L of sulfamethoxazole and 1.6–12.8 mg/L of trimethoprim). A control sample with no antibiotic at Days 0 and 15 for each experiment was used. The minimum inhibitory concentration (MIC) was defined as the first antibiotic concentration resulting in inhibition of growth.

Growth of the six isolates on HEL cells without antibiotic was quantified at Day 15. For the six strains, the mean growth ratio was 1.67 (range 1.42–1.84) and the mean estimated doubling time was 21 h. Quantification of growth with doxycycline and minocycline at Day 15 revealed an inhibition of growth for MICs between 0.25 mg/L and 0.5 mg/L for the six strains (Fig. 1). All strains were susceptible to levofloxacin (MICs of 0.1–0.5 mg/L), sulfamethoxazole/trimethoprim (MICs of 8/1.6 mg/L), rifampicin (MICs of 0.25–0.5 mg/L) and tigecycline (MICs of 0.25 mg/L) (Fig. 1). Considering macrolides, the six strains were resistant to erythromycin and azithromycin (MICs ≥ 8 mg/L). One strain isolated from the blood of a patient with pneumonia was resistant to telithromycin (MIC > 8 mg/L).

We describe the antibiotic susceptibility of strains of a genotype MST 17 clone of *C. burnetii* from French Guiana using flow cytometry. Genotype 17 isolates are all susceptible to doxycycline. This is an important point since resistance to doxycycline has been previously described in *C. burnetii* strains [4]. The French Guianian strains in this study were also all susceptible to sulfamethoxazole/trimethoprim, suggesting that this agent can be used in French Guiana in case of Q fever during pregnancy, as recommended in metropolitan France [5]. Interestingly, we describe for the first time a strain resistant to telithromycin [4]. Although resistance to macrolides has been well described in *C. burnetii*, telithromycin was an exception and considered before this study as a potential useful agent in Q fever [4]. Except for this strain, the antibiotic susceptibility profile of genotype 17 strains is homogeneous and no important difference appears between strains causing endocarditis compared with strains isolated from vascular infections or pneumonia. These results suggest that doxycycline remains the agent of choice in the first-line treatment of Q fever pneumonia in French Guiana.

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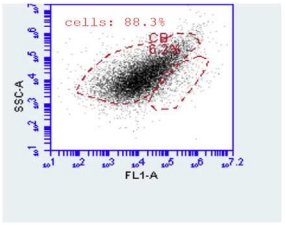

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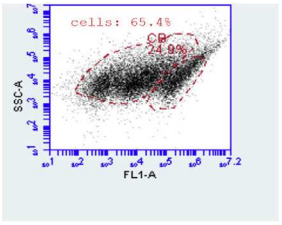

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STRAIN ORIGIN

ANTIBIOTIC	Cardiac valve Endocarditis 1	Cardiac valve Endocarditis 2	Blood Vascular aneurysm infection 3	Blood Pneumonia 4	Blood Pneumonia 5	Blood Pneumonia 6
Doxycycline	0.25	0.25	0.25	0.25	0.25	0.25
Minocyclin	0.25	0.5	0.25	0.25	0.25	0.25
Levofloxacin	0.5	0.5	0.5	0.5	0.1	0.5
Sulfamethoxazole Trimethoprim	8/1.6	8/1.6	8/1.6	8/1.6	8/1.6	8/1.6
Rifampicin	0.25	0.25	0.25	0.5	0.25	0.25
Erythromcycin	>8	8	>8	8	8	>8
Azithromycin	>8	>8	>8	>8	>8	>8
Telithromycin	2	2	4	4	2	>8
Tigecycline	0.25	0.25	0.25	0.25	0.25	0.25

Fig. 1. (A) Gating of HEL cells only due to FL1-A intensity and SSC. (B) Gating of *Coxiella burnetii* (CB) on HEL cells due to FL1-A intensity and SSC. (C) Table of MICs (mg/L) of antibiotics against the six strains of *C. burnetii* from French Guiana (MICs defined as resistant in red). FL1, fluorescence detector; SCC, side scatter; MIC, minimum inhibitory concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

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Competing interests

None declared.

Ethical approval

Not required.

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Partie IV : Les manifestations cliniques de l'infection à *C. burnetii :* diagnostic et traitement

AVANT PROPOS

Le diagnostic des infections persistantes à *C. burnetii* peut être fastidieux. D'autre part, le terme de fièvre Q chronique, basé sur une définition sérologique n'est pas satisfaisant, car il laisse de côté la problématique de la localisation du foyer infectieux. Les localisations principales des infections persistantes à *C. burnetii* sont les endocardites et les infections vasculaires (12, 13). Cependant, d'autres foyers infectieux tels que des infections ostéoarticulaires, des adénopathies, ou des pseudotumeurs pulmonaires ont été décrites (14–17). Par ailleurs, il a récemment été montré que les lymphadénites à *C. burnetii* pouvait prédisposer à l'évolution vers un lymphome (18). Cependant, dans certains cas, il existe des symptômes cliniques persistants (à type de fièvre ou altération de l'état général), associés à une sérologie positive en IgGI, sans qu'il soit possible aux cliniciens d'identifier une localisation infectieuse.

Le TEP scanner est un outil d'imagerie fonctionnelle corps entier, qui repose sur l'injection d'un traceur radioactif le 18 F-FDG, qui va se fixer au niveau de foyers organiques hypermétaboliques (i.e, consommant une grande quantité de glucose). Les indications de cet examen sont en plein essor ces dernières années. Initialement utilisé en oncologie, il est maintenant indiqué notamment dans le bilan étiologique des fièvres prolongées. Concernant la fièvre Q, le TEP scanner fait désormais partie des critères diagnostiques des endocardites et des infections vasculaires (19). Cependant, cette classification repose sur de petites cohortes de patients et cet examen n'a jamais été évalué dans la détection d'autres foyers infectieux causés par *C. burnetii*.

Nous avons réalisé une analyse rétrospective des patients suivis dans notre centre pour une infection à *C. burnetii* avec pour objectif principal de décrire les différents foyers infectieux retrouvés par cet examen. Notre deuxième objectif était d'évaluer si le TEP scanner permettait

de mettre en évidence une localisation infectieuse, dans les cas d'errance diagnostique avec anticorps IgG de phase I élevés.

Un total de 167 patients avec une infection à *C. burnetii* et ayant bénéficié d'un TEP scanner ont été inclus. Quatre vingt dix neuf patients (59%) avaient un TEP scanner positif avec au moins un foyer hypermétabolique retrouvé. Nous avons retrouvé 21 foyers hypermétaboliques ostéoarticulaires et dans 8 cas cette localisation était unique. Nous avons retrouvé 27 foyers ganglionnaires, 21 foyers valvulaires et 26 foyers vasculaires. Onze patients présentaient un hypermétabolisme de la moelle osseuse et 9 avaient un hypermétabolisme pulmonaire. D'autre part, le TEP scanner a permis un changement de diagnostic (en découvrant ou confirmant un foyer infectieux) chez 62 patients parmi les positifs (62,5%). Concernant les patients qui présentaient des IgG de phase I élevés sans hypothèse sur le foyer infectieux impliqué, la localisation retrouvée le plus fréquemment était la localisation ostéoarticulaire, suivie par les lymphadénites et enfin les endocardites et les infections vasculaires.

Ce travail représente à ce jour la plus importante cohorte de patients atteints de fièvre Q ayant bénéficié d'un TEP scanner. Notre étude montre de façon étonnante une proportion importante de localisations ostéoarticulaires, alors que cette entité avait été décrite de manière anecdotique auparavant dans la littérature. Il en va de même pour les localisations ganglionnaires. Nous proposons donc en conclusion de ce travail de nouveaux scores diagnostiques définissant ces deux entités cliniques.

Dans un deuxième travail, nous avons étudié les facteurs pronostiques dans l'évolution des infections vasculaires à *C. burnetii*. Ces infections surviennent principalement chez des patients présentant des facteurs de risque tels qu'un anevrysme vasculaire préexistant ou une prothèse vasculaire. Sur ce terrain, leur pronostic est extrêmement péjoratif avec des taux de mortalité entre 18 et 25% en fonction des séries. Peu d'études existent sur les modalités de

traitement de ces infections et notamment sur la place de la chirurgie. La décision de traitement chirurgical est souvent difficile à prendre du fait de localisations vasculaires à risque et du risque anesthésique chez des patients présentant fréquemment de nombreux facteurs de risque cardiovasculaires. On trouve dans la littérature des cas qui décrivent une évolution favorable après traitement chirurgical et une étude rétrospective sur 40 patients suggère un bénéfice de la chirurgie en terme de survie à 3 ans, quand celle-ci est associée à l'antibiothérapie recommandée (doxycycline et hydroxychloroquine) (13, 20). A l'inverse, une étude récente réalisée aux Pays-Bas suggère que la chirurgie est associée à un taux de mortalité plus important dans la catégorie « fièvre Q chronique » (21).

Dans ce travail, nous rapportons dans un premier temps le cas d'un patient de 34 ans atteint d'une infection de prothèse aortique. Au de la localisation chirurgicale délicate, il a été initialement récusé à la chirurgie et son état s'est aggravé sous traitement médical seul, avec apparition de nombreuses localisations septiques secondaires (abcès rénaux, pulmonaires et spléniques). Ce patient a finalement consulté un chirurgien exerçant à Liverpool qui a réalisé un débridement chirurgical associé à un changement de prothèse vasculaire. L'évolution a secondairement été extrêmement favorable avec une division par 5 des titres sérologiques d'IgG de phase I à 4 mois de cette prise en charge. A 4 ans de la prise en charge, ce patient est guéri. A partir de ce cas, nous avons donc décidé d'analyser rétrospectivement le devenir en terme de survie et d'évolution sérologique des patients atteints d'infections vasculaires à *C. burnetii* (avec ou sans prothèse) diagnostiqués dans notre centre (Centre National de Reference de la fièvre Q) sur les 29 dernières années, en fonction de la présence ou non d'un traitement chirurgical.

Le premier de nos résultats est que l'incidence moyenne des infections vasculaires à *C. burnetii* diagnostiquées sur les cinq dernières années a nettement augmentée (8,8 cas par an, vs 3,5 cas par an avant cette période, $p<0,001$), ce qui est probablement dû à une évolution dans

les critères diagnostiques de ces infections et à l'apparition de nouveaux outils tels que le TEP scanner. Parmi les 86 patients atteints d'infections vasculaires, 58 avaient une infection de prothèse et 28 avaient une infection d'anevrysme sans prothèse. Le taux de mortalité à 2 ans et demi de suivi était de 18%, ce qui est comparable au taux retrouvés dans les études antérieures. Le taux de mortalité dans le groupe des patients non opérés était significativement plus élevé que dans le groupe des patients opérés. (93,5% versus 71,4%, p=0,02). En analyse de Kaplan Meier et de Cox, la chirurgie était le seul facteur prédictif de survie et était également associée à une évolution sérologique favorable. Néanmoins, dans le sous-groupe des infections sur prothèse vasculaires ce bénéfice de la chirurgie sur la survie n'était pas retrouvé, mais il existait un bénéfice en terme sérologique. Ceci s'explique potentiellement par le fait que les patients porteurs de prothèse vasculaires ont des facteurs de risque anesthésiques plus lourds, entraînant une surmortalité per et post opératoire.

Etant donné le mauvais pronostic de ces infections, nous proposons une stratégie de prévention par le dépistage systématique des anevrysme vasculaires chez les patients de plus de 65 ans présentant une infection à *C. burnetii*, ce qui permettrait de débuter une antibioprophylaxie comme cela est recommandé dans les endocardites.

Article 6 : 18F-FDG PET/CT as a central tool in the shift from chronic Q fever to *Coxiella burnetii* persistent focalized infection: A consecutive case series.

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^{18}F-FDG PET/CT as a central tool in the shift from chronic Q fever to *Coxiella burnetii* persistent focalized infection

A consecutive case series

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Abstract

Because Q fever is mostly diagnosed serologically, localizing a persistent focus of *Coxiella burnetii* infection can be challenging. ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) could be an interesting tool in this context.

We performed a retrospective study on patients diagnosed with *C burnetii* infection, who had undergone ^{18}F-FDG PET/CT between 2009 and 2015. When positive ^{18}F-FDG PET/CT results were obtained, we tried to determine if it changed the previous diagnosis by discovering or confirming a suspected focus of *C burnetii* infection.

One hundred sixty-seven patients benefited from ^{18}F-FDG PET/CT. The most frequent clinical subgroup before ^{18}F-FDG PET/CT was patients with no identified focus of infection, despite high IgG1 serological titers (34%). For 59% (n = 99) of patients, a hypermetabolic focus was identified. For 62 patients (62.6%), the positive ^{18}F-FDG PET/CT allowed the diagnosis to be changed. For 24 of them, (38.7%), a previously unsuspected focus of infection was discovered. Forty-two (42%) positive patients had more than 1 hypermetabolic focus. We observed 21 valvular foci, 34 vascular foci, and a high proportion of osteoarticular localizations (n = 21). We also observed lymphadenitis (n = 27), bone marrow hypermetabolism (n = 11), and 9 pulmonary localizations.

We confirmed that ^{18}F-FDG PET/CT is a central tool in the diagnosis of *C burnetii* focalized persistent infection. We proposed new diagnostic scores for 2 main clinical entities identified using ^{18}F-FDG PET/CT: osteoarticular persistent infections and lymphadenitis.

Abbreviations: ^{18}F-FDG PET/CT = ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography, *C burnetii* = *Coxiella burnetii*.

Keywords: ^{18}F-FDG PET/CT, *Coxiella burnetii*, diagnosis, focalized persistent infection, Q fever

1. Introduction

Q fever is a worldwide zoonosis caused by the bacterium *Coxiella burnetii*. Since the first studies on Q fever, a dichotomy has been established between "acute Q fever" and "chronic Q fever."[1] The term chronic Q fever was used due to the inability to determine the infected site in patients with persistent symptoms or a positive serology with an increase in phase I IgG, suggesting

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CE and CM equally contributed to the work and should be considered as co-first authors.

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an active infection.[2] The term "chronic Q fever" is, however, misleading because it combines many different clinical entities under serological criteria.[3] Serological cut-offs alone are not sufficient to determine the persistence of *C burnetii* infection. This phenomenon is illustrated by the Q fever epidemic in French Guiana, where patients with primary Q fever presented high levels of phase I IgG with no systematic clinical progression towards a persistent focalized infection.[4] In France, *C burnetii* infection is endemic, but localized outbreaks and hyperendemic foci are described .[5] The disease is more often diagnosed in the Southeast of France where the French National Referral Center for Q fever is located.[5]

Endocarditis and vascular infections represent the majority of the described focalized persistent infections.[6,7] Several other localizations have been described, but less frequently, such as joint and bone infections,[8,9] lymphadenitis,[10] pericarditis, lung pseudo-tumor, and gall bladder infection.[11] In the case of endocarditis and vascular infections, definition scores have been elaborated, in which the ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) helps detecting infection focus (Table 1).[3] Thanks to an early diagnosis strategy, prophylaxis, and treatment, the prognosis of *C burnetii* endocarditis has drastically changed in our center.[6,12] The mortality rate has fallen from 60% to 5%.[6] However, vascular infections remain a very severe entity, with high mortality rates (up to 25%) and requiring surgical treatment.[7] In *C burnetii* joint and lymph node infections, very little is known about prognosis and treatment.[8–10] These differences in prognosis and treatment between the types of focalized Q fever infections illustrate the inaccuracy of grouping them under the global term of "chronic Q fever."

Nonetheless, in some circumstances, clinical symptoms and/or high IgGI antibodies persist without evident focus of infection. Physicians are confronted with therapeutic challenge, which is whether to treat a potentially fatal infection without knowing the site of infection or not. Moreover, classical morphological tools often fail to identify *C burnetii* infection because anatomical changes can be very slight. For example, in *C burnetii* persistent endocarditis, typical vegetation is observed in only 30% of cases, and echocardiography detected a valvular insufficiency in 75% of cases.[6] Vascular infections can be revealed only by aneurysm or vascular graft rupture.[13]

^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography is an imaging modality that allows measurement of metabolic activity within an organ, obtained from the emission of positrons after disintegration of the injected radioactive product. As the majority of the malignant cells have high glycolytic activity, detection of their hypermetabolism was first used in clinical oncology.[14] Recently, it has been used for the identification of inflammatory and infectious processes because they also result in significant FDG uptake by the inflammatory cells. ^{18}F-FDG PET/CT has been used for the detection and monitoring of fever of unknown origin (FUO) and in a growing number of infections.[15,16] Regarding *C burnetii*, around 10 references are found in the literature reporting the use of ^{18}F-FDG PET/CT. Among these references, Barten et al reported 15 patients with *C burnetii* endocarditis and vascular infections.[17] Other reports describe hepatic, bone marrow, lymphadenitis, articular, and prostatic uptake of ^{18}F-FDG PET/CT.[8,17–23]^{18}F-FDG PET/CT has been included as a criterion in the definition scores for *C burnetii* endocarditis, articular prosthesis, and vascular infections. However, this definition was based on a very limited number of patients, and its utility in detection of other foci of infection has not been assessed.

Herein, our objective was to describe the different foci that could be detected in patients with persistent *C burnetii* infection. Thanks to this description, our secondary objective was to assess if ^{18}F-FDG PET/CT allowed the detection of a focus of infection in patients with unlocalized persistent *C burnetii* infection.

2. Patients and methods

2.1. Case definition

The French National Reference Center for Q fever receives samples for *C burnetii* testing[4] from the entire country. Between January 2009 and June 2015, 1555 patients were tested positive for Q fever in our center. Clinical and laboratory data were collected prospectively for all patients—thanks to a standardized questionnaire. For patients who did not benefit from a medical monitoring by our center in our center, data were collected over the phone to complete the standardized questionnaire.

All patients with an active *C burnetii* infection who benefited from a ^{18}F-FDG PET/CT were included in our study (Fig. 1 and eFig. 1, http://links.lww.com/MD/B217). Among these patients, several subgroups were identified and differentiated according to the diagnosis before ^{18}F-FDG PET/CT: primary *C burnetii* infection was defined by the association of clinical symptoms (fever and/or hepatitis and/or pneumonia) with serologic criteria for phase II IgG levels $\geq$200 and phase II IgM levels $\geq$50, or by a Polymerase Chain Reaction (PCR) and no endocarditis. Possible or definite *C burnetii* endocarditis, vascular infection, and joint prosthesis infection were defined according to the recent criteria (Table 1).[3,8] The rest of the cases were patients with persistent elevated phase I IgG ($\geq$800) for more than 3 months without any focus of infection at clinical examination and transthoracic echocardiography.

We excluded patients for whom ^{18}F-FDG PET/CT was performed before the onset of symptoms that motivated the serology. Patients with serology indicative of a past resolved *C burnetii* infection were also excluded, and ^{18}F-FDG PET/CT examinations that were performed for follow-up were excluded (Fig. 1).

The study was approved by the local ethics committee (Comité de Protection des Personnes Sud Mediterranée 1). All patients gave informed consent.

2.2. Diagnosis of Coxiella burnetii infection

We used an indirect immunofluorescence assay to quantify IgG, IgM, and IgA titers against phase I and phase II, as previously described .[24] DNA was extracted using the QIAamp Tissue Kit (QIAGEN GmbH, Hilden, Germany), and these extracts were used as templates for PCR amplification as previously described.[25] Culture, immunohistochemistry, and fluorescent in situ hybridization (FISH) targeting *C burnetii* 16S rRNA were performed.[10,25]

2.3. ^{18}F-FDG PET/CT

^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography was performed in the fasting state for at least 6 hours and the glucose level was lower than 150 mg/dL. An FDG dose of 4 to 5 MBq/kg was administered intravenously and imaging was performed 60 minutes after injection in accordance with each center's protocol. The images were analyzed visually

Table 1

Definition criteria for *C burnetii* endocarditis, vascular infections, and prosthetic joint arthritis.

Definition of Q fever endocarditis according to Raoult, 2012[3]	Definition of Q fever vascular infection according to Raoult, 2012[3]	Definition of *C burnetii*-related prosthetic joint arthritis according to Million, 2014[8]
Definite criterion:	Definite criterion:	Definite criterion:
Positive culture, PCR, or immunochemistry of a cardiac valve	Positive culture, PCR, or immunochemistry of an arterial samples (prosthesis or aneurism) or a periarterial abscess or a spondylodiscitis linked to aorta	Positive culture, polymerase chain reaction, or immunochemistry of a periprosthetic biopsy or joint aspirate
Major criteria	Major criteria	Major criteria
Microbiology: positive culture or PCR of the blood, an emboli or serology with IgG1 antibody titer ≥6400	Microbiology: positive culture, PCR of the blood or emboli, or serology with IgGI antibodies ≥6400	Microbiology
Evidence of endocardial involvement	Evidence of vascular involvement:	Positive culture or polymerase chain reaction of the blood
Echocardiogram positive for IE: oscillating intracardiac mass on valve or supporting structures, in the path of regurgitant jets or on implanted material in the absence of an alternative anatomic explanation; or abscess; or new partial dehiscence of a prosthetic valve; or new valvular regurgitation (worsening or changing of pre-existing murmur is not sufficient)	CT scan: aneurism or vascular prosthesis + periarterial abscess, fistula, or spondylodiscitis	Positive *C burnetii* serology with IgGI antibodies ≥6400
PET scan displaying a specific valve fixation and mycotic aneurism	PET scan specific fixation on an aneurism or vascular prosthesis	Evidence of prosthetic involvement:
		Computed tomography scan or MRI positive for prosthetic infection: collection or pseudo-tumor of the prosthesis
		Positron emission tomography scan or indium leukocyte scan showing a specific prosthetic hypermetabolism consistent with infection†
Minor criteria	Minor criteria	Minor criteria
Predisposing heart condition (known or found on echography)	Serological IgGI ≥800 <6400	Presence of a joint prosthesis (indispensable criteria)
Fever, temperature >38°C	Fever, temperature ≥38°C	Fever, temperature >38°C
Vascular phenomena, major arterial emboli, septic pulmonary infarcts, mycotic aneurysm (observed during PET scan), intracranial hemorrhage, conjunctival hemorrhages, and Janeway lesions.	Emboli	Joint pain
Immunologic phenomena: glomerulonephritis, Osler nodes, Roth spots, or rheumatoid factor	Underlying vascular predisposition (aneurism or vascular prothesis)	Serologic evidence: positive *C burnetii* serology with IgGI antibodies ≥800 and <6400 mg/dL
Serological evidence: IgG1 antibody titers ≥800 <6400		
Diagnosis definite	Diagnosis definite	Diagnosis definite
1A criterion	A criterion	1A criterion
2B criteria	2B criteria	2B criteria
1B criterion and 3C criteria (including 1 microbiological characteristic and a cardiac predisposition)	1B criterion and 2C criteria (including 1 microbiological characteristic and a vascular predisposition)	1B criterion and 3C criteria (including 1 piece of microbiology evidence and presence of a joint prosthesis)
Possible diagnosis	Possible diagnosis	Possible diagnosis
1B criterion and 2C criteria (including 1 microbiological characteristic and a cardiac predisposition)	Vascular predisposition, serological evidence, and fever or emboli.	1B criterion, 2C criteria (including 1 piece of microbiology evidence and presence of a joint prosthesis)
3C criteria (including 1 microbiological characteristic and a cardiac predisposition)		3C criteria (including positive serology and presence of a joint prosthesis)

C burnetii = *Coxiella burnetii*, PCR = polymerase chain reaction, PET = positron emission tomography, MRI = magnetic resonance imaging.

and semiquantitatively by measuring the maximum standardized uptake value (SUV-max). Hypermetabolic ^{18}F-FDG activity was considered as a potential site of infection when it did not correspond to physiological uptake (myocardial, liver, bladder, ureter, kidney, and gastrointestinal foci). ^{18}F-FDG PET/CT was performed in several centers without a common interpretation.

When the ^{18}F-FDG PET/CT was performed in another center, the protocol for ^{18}F-FDG PET/CT, images, and interpretation were collected retrospectively. When images were not available, reports alone were collected.

2.4. Main outcome: change of diagnosis after 18-FDG PET/CT

We considered that the ^{18}F-FDG PET/CT results allowed the diagnosis to be changed when a previously unknown localization

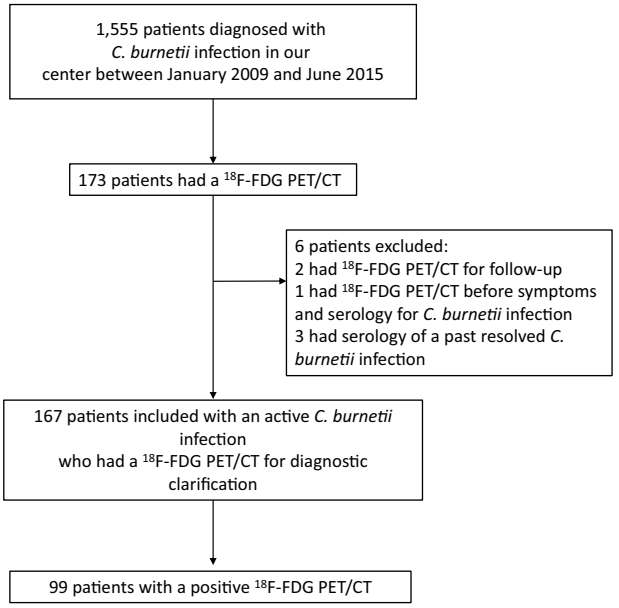

Figure 1. Flow chart.

of the infection was discovered, or when a possible endocarditis or vascular infection was confirmed.

2.5. Statistical analysis

Descriptive statistics for continuous variables are represented as median. Categorical variables are reported in terms of the number and percentages of patients affected. Variables were calculated using SPSS 22 Statistics Software.

3. Results

One hundred sixty-seven patients with *C burnetii* active infection had a ^{18}F-FDG PET/CT performed, including 37 women (22%) and 130 men (78%). The mean age of patients was 58.4 ± 16 years. The type of *C burnetii* active infection before ^{18}F-FDG PET/CT were: persistent elevated phase I IgG for more than 3 months for 57 patients (34%), possible endocarditis for 39 patients (23%), definite endocarditis or vascular infection for 31 patients (19%), primary Q fever for 25 patients (11.3%), possible vascular infection for 14 patients (8%), and possible osteoarticular infection for 1 patient (0.5%).

^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography revealed positive hypermetabolism for 99 patients (59%). Fifty-seven of these patients (34.7% of all patients) had 1 hypermetabolism, 42 patients (15%) had 2 hypermetabolic foci, 10 (6%), and 3 patients (1.8%), respectively, had 3 and 4 hypermetabolic foci. The highest number of infectious foci located in 1 person was 5, which were found in 3 patients.

3.1. Osteoarticular localizations

Osteoarticular hypermetabolism was identified in 21 patients (Fig. 2 and Table 2). Osteoarticular localizations as the main focus of infection were observed in 8 cases (Fig. 2, Tables 2 and 3). Three infections involved a joint prosthesis. For 2 patients, we observed an acromioclavicular hypermetabolism, and 1 other patient had shoulder involvement (Figs. 2 and 3). One patient had tenosynovitis and another had an isolated spondylodiscitis.

Thirteen osteoarticular hypermetabolisms were associated with other hypermetabolic foci. In this context, we found a majority of spondylodiscitis (n = 9) complicating endocarditis or vascular infection (Table 3).

3.2. Lymphadenitis

Lymphadenitis hypermetabolism was identified in 27 patients (Figs. 2 and 3). Lymphadenitis was the sole focus for 11 patients, among which 7 (25%) also presented a primary *C burnetii* infection, and the remaining 4 were cases of isolated persistent lymphadenitis (14.8%). Lymphoma was diagnosed in 2 patients with lymphadenitis hypermetabolism.

Lymphadenitis hypermetabolism was associated with another persistent focalized infection in 16 cases (59%), with 5 patients presenting 3 or more concomitant foci. Cardiovascular foci were present in 5 cases (1 endocarditis, 4 vascular infections), osteoarticular foci in 6 cases (22%), and other foci are detailed in Table 2.

3.3. Endocarditis

A total of 21 patients (21%) showed a hypermetabolism suggesting endocarditis, including 6 hypermetabolisms on a native valve, 14 hypermetabolisms on a prosthetic valve, and 1 hypermetabolism on a pacemaker (Figs. 2 and 3). Before the ^{18}F-FDG PET/CT, these patients had possible endocarditis (n = 13), definite endocarditis (n = 3), persistent IgG1 (n = 3), suspicion of osteoarticular infection (n = 1), and suspicion of vascular infection (n = 1).

3.3.1. Endocarditis with aortic hypermetabolism and other embolic localizations. Eight patients with endocarditis had a simultaneous aortic hypermetabolism (6 Bentalls and 2 mycotic aneurysms) (Table 2). One of these patients had an associated spondylodiscitis and psoas abscess. One patient had a simultaneous spondylodiscitis and 3 had other articular foci.

3.4. Vascular infections

Twenty-six patients (26%) had a vascular hypermetabolism without endocarditis. Four of these patients had associated hyperfixating spondylodiscitis and psoas abscesses (Figs. 2 and 3). Diagnosis subgroups before^{18}F-FDG PET/CT were: possible vascular infections in 11 cases, definite vascular infections in 4 cases, possible endocarditis in 3 cases, definite endocarditis in 3 cases, persistent IgGI in 3 cases, and primary Q fever in 2 cases.

3.5. Bone marrow

Eleven patients presented an increased bone marrow uptake (Figs. 2 and 3). Among them, 4 presented a primary Q fever infection, 6 had a persistent cardiovascular focalized infection, 1 had an osteoarticular infection, 5 had a concomitant spleen hypermetabolism, and 4 had a concomitant lymphadenitis uptake. Four patients presented bone marrow uptake as the unique hypermetabolic focus (2 in a context of primary infection and 2 associated with nonhypermetabolic possible and definite endocarditis).

3.6. Pulmonary localization

For 9 patients, we observed a pulmonary hypermetabolism (Fig. 3). Five patients displayed conventional lobar pneumonia, and 2 had a hypermetabolic nodule. Four of them had a ^{18}F-FDG PET/CT in a context of primary Q fever (Table 2).

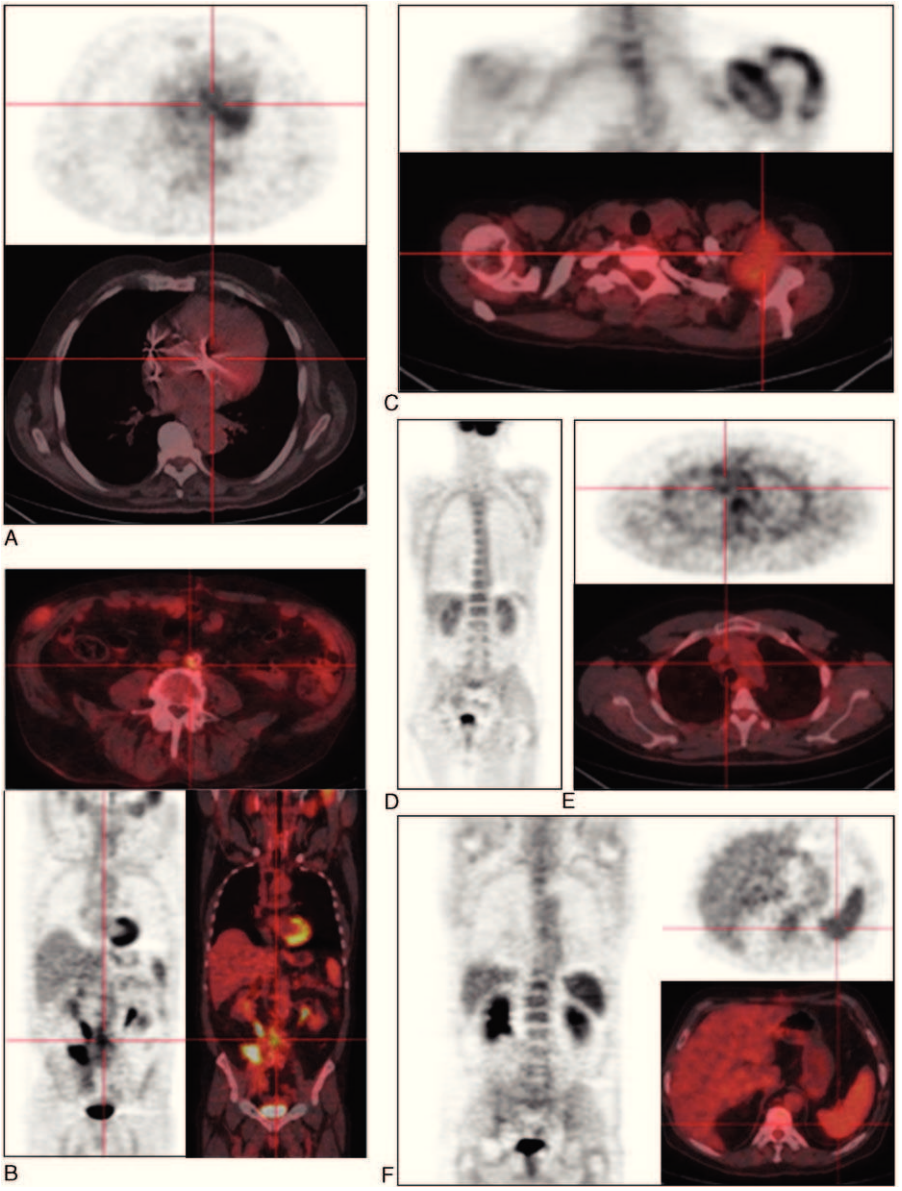

Figure 2. Hypermetabolic foci of *Coxiella burnetii* infection identified by ^{18}F-FDG PET/CT. A, Aortic valve hypermetabolism during definite Q fever endocarditis; B, abdominal aortic hypermetabolism during definite Q fever vascular infection; C, bursitis, arthritis foci during Q fever osteoarticualr infection; D, bone marrow hypermetabolism during Q fever; E, Q fever lymphadenitis identified with PET scan; F, spleen hypermetabolism during Q fever. ^{18}F-FDG PET/CT = ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography.

3.7. Other hypermetabolic foci

We observed the following other hypermetabolic foci: prostatic (5 patients), thyroid (4 patients), and laryngeal (4 patients). These foci were always associated with another main focus of infection.

3.8. Clinical relevance of ^{18}F-FDG PET/CT in the localization of Coxiella burnetii persistent focalized infection

Positive ^{18}F-FDG PET/CT allowed the diagnosis to be changed for 62 patients (62.6%). When the following 2 groups of patients were pooled, the first group being patients with isolated persistent elevated IgGI for more than 3 months and the second group being patients with possible endocarditis (n = 96), the diagnosis was changed in 55% of the patients—thanks to ^{18}F-FDG PET/CT. For patients with persistent isolated IgG1 (n = 57), the most frequent entities were an osteo-articular infection focus (n = 8, 30.7%) (Table 4) and lymphadenitis (n = 7, 26.9%) followed by endocarditis (n = 3), vascular infections (n = 3), lung pseudotumor (n = 2), and pulmonary hypermetabolism evocative of primary infection (n = 1; Figs. 2 and 3, Table 4). Six definite vascular infections were discovered in a context of suspicion of endocarditis (Table 4).

4. Discussion

We here report the largest case series of Q fever patients benefiting from a ^{18}F-FDG PET/CT. The mean age of patients (58 years) and the male predominance is concordant with the classic epidemiology of symptomatic Q fever.[26] More than half of these patients showed a positive ^{18}F-FDG PET/CT, and this examina-

Table 2

Description of 18F-FDG foci.

PET foci	Cardiac valve	Vascular	Osteoarticular	Lymphadenitis	Bone marrow	Pulmonar
N = positive ^{18}F-FDG PET/CT (% of total patients)	21 (12.5%)	34 (20.35%)	21 (12.5%)	27 (16%)	11 (6.5%)	9 (0.05%)
Age (mean)	63.6	65.8	64.95	60.83	52.7	57.81
Sex (M) (%)	17 (81%)	31 (91%)	19 (90%)	22 (81.5%)	9 (72%)	6 (66%)
IgG I (median)	2400	1200	600	800	200	800
IQR 25% percentile	800	700	400	400	100	25
IQR 75% percentile	12800	16000	1600	3200	800	2000
Associated hypermetabolism						
None	8	13	5	11	4	6
Endocarditis	ALL	8	2	1	0	0
Vascular infection	8	ALL	5	4	2	1
Osteoarticular	4	7	ALL	6	1	0
Bone marrow	0	3	1	4	ALL	1
Lymphadenitis	1	4	6	ALL	4	1
Spleen	2	4	1	4	5	1
Lung	0	1	0	1	1	ALL
Prostate	1	3	0	2	0	0

^{18}F-FDG PET/CT = ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography, IQR = interquartile range.

tion allowed the diagnosis to be changed in 62.6% of cases. Regarding hypermetabolism, it is that a high proportion of patients (42%) present 2 or more foci of fixation, reflecting the systemic nature of the *C burnetii* infection.

Because no gold standard imaging technique exists in the detection of *C burnetii* foci of infection, no statistical comparison could be made to assess the sensitivity and specificity of ^{18}F-FDG PET/CT, and this represents 1 limitation of our study. Patients diagnosed in our center may be followed in other cities, so that no common interpretation of ^{18}F-FDG PET/CT results was performed. This is another limitation of our study.

For patients with persistent elevated IgGI levels, we observed a focus of infection in 38.7% of cases. One striking finding is that the majority of these patients had an osteoarticular focus of infection (33%). This is an important result since osteoarticular Q fever infections have been considered to be rare occurrences, representing about 2% of Q fever cases.[27] The most widely reported localizations in the literature were osteomyelitis[20,28]

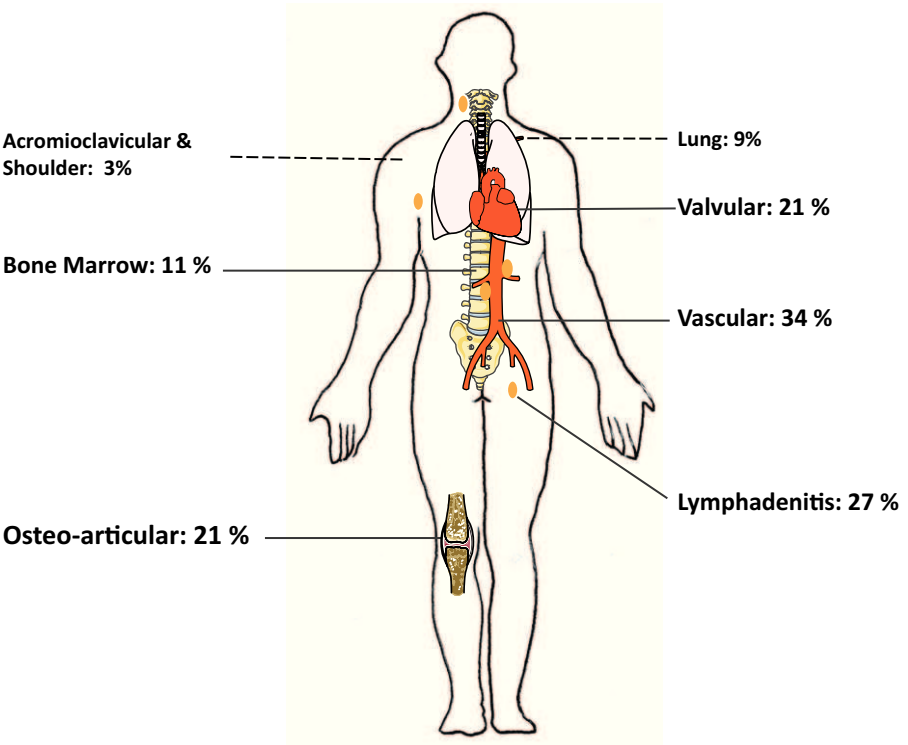

Figure 3. Distribution of Q fever foci identified by ^{18}F-FDG PET/CT. ^{18}F-FDG PET/CT = ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography.

Table 3

Details of the 8 patients with osteoarticular hypermetabolism as the main focus of infection.

Patient	Sex	Age	Indication for ^{18}F-FDG PET/CT	IgGI titer	Localization	Associated hypermetabolism
1	M	43	Isolated elevated IgGI	3200	Knee prosthesis	Bone marrow and lymph node
2	F	46	Isolated elevated IgGI	3200	Left shoulder	Contiguous lymph node
3	M	61	Isolated elevated IgGI	1600	Tibial tenosynovitis	None
4	M	56	Acute Q fever with bad evolution	3200	Acromio clavicular	Contiguous lymph node
5	M	68	Acute Q fever with bad evolution	25,600	Acromio clavicular	Contiguous lymph node
6	M	85	Isolated elevated IgGI	6400	Hip prosthesis	Contiguous lymph node
7	M	78	Isolated elevated IgGI	3200	Hip prosthesis	Mediastinal lymph node
8	M	80	Isolated elevated IgGI	800	Spondylodiscitis	No

^{18}F-FDG PET/CT = ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography.

and isolated spondylodiscitis.[29] Two cases of tenosynovitis of the wrist[29] and Q fever infections of a joint prosthesis have been reported.[8,30] We found only 1 case of isolated spondylodiscitis. All other cases of spondylodiscitis were associated with vascular infections or endocarditis. This result confirms that isolated *C burnetii* spondylodiscitis is quite rare. We observed 2 cases of acromioclavicular hypermetabolism with contiguous lymphadenopathy. Only 1 similar case of Q fever subacromial bursitis has been reported.[9] These 2 additional cases suggest a new Q fever clinical entity. We also reported here the fourth case of *C burnetii* tenosynovitis.[9,29] Thus, we suggest a new definition score for *C burnetii* osteoarticular infections (Table 5, part I). Definite criteria for diagnosis are microbiological proof (by PCR, culture, FISH, or immunohistochemistry) of infection in a bone or joint biopsy or joint fluid aspirate. Major and minor criteria are detailed in Table 5 (part I). Definite diagnosis of Q fever osteoarticular infection is defined by the presence of either 1 definite criterion, 2 major criteria, or 1 major and 3 minor criteria (Table 5, part I).

Q fever lymphadenitis was described in the literature as a proven microbiological focus of Q fever. *C burnetii* was identified within lymph nodes by PCR, immunohistochemistry, and FISH (eFig. 2, http://links.lww.com/MD/B217).[31] The use of ^{18}F-FDG PET/CT, however, has been anecdotally described in this setting. In a recent study, we described 59 cases of lymphadenitis associated with *C burnetii* infection, among which 42% were associated with persistent focalized infection.[31] Moreover, we recently demonstrated that *C burnetii* may predispose to lymphomagenesis.[31]^{18}F-FDG PET/CT is therefore a tool of choice for monitoring *C burnetii* lymphadenitis. Thus, we suggest a diagnostic score for *C burnetii* persistent lymphadenitis (Table 5, part II). *C burnetii* lymphadenitis is definite when the bacteria have been identified within lymph nodes by culture, PCR, immunohistochemistry, or FISH, or when 2 major criteria are fulfilled.

Bone marrow uptake was observed in both primary and persistent focalized infection and was associated in almost 50% of cases with spleen hypermetabolism, reflecting the lymphoid tropism of *C burnetii*. Bone marrow involvement during Q fever has been reported in cases of pancytopenia, hemophagocytic syndrome with aspects of doughnut granuloma,[18,32] and has also recently been described as a diffuse bone marrow ^{18}F-FDG PET/CT hypermetabolism.[19,33]

As mentioned in the literature, we found that ^{18}F-FDG PET/CT is particularly useful in the diagnosis of prosthetic valve endocarditis.[34] Of 21 patients with positive valvular ^{18}F-FDG PET/CT hypermetabolism, over two-thirds had a prosthetic cardiac valve. We described 1 case with pacemaker hypermetabolism. In over two-thirds of cases (71%), valvular hypermetabolism required us to change the diagnosis by confirming or revealing an endocarditis. This is particularly interesting in *C burnetii* endocarditis, where typical echocardiography findings such as vegetations are frequently lacking.[2] One-third of patients presented associated vascular or osteoarticular foci, supporting the usefulness of ^{18}F-FDG PET/CT in the detection of extracardiac complications of infective endocarditis.[35]

Thanks to ^{18}F-FDG PET/CT, we detected 34 vascular foci, 15 of them involving a vascular prosthesis (44%). In 6 cases, these

Table 4

Cases of change in diagnosis after 18F-FDG PET/CT.

	Diagnosis after 18F-FDG PET/CT							
Diagnosis before 18F-FDG PET/CT	Primary infection	Lymphadenitis	Definite endocarditis	Definite Vascular infection	Osteoarticular infection	Lung pseudotumor	Lymphoma	Total
Primary infection		3		2				5
IgGI ≥800 >3 mos	1	7	3	3	8	2		24
Possible endocarditis	1		13	3				17
Possible native vascular infection				11				11
Possible spondylodiscitis			1					1
Definite endocarditis				3				3
Definite native vascular infection							1	1
Total	2	10	17	22	8	2	1	62

^{18}F-FDG PET/CT = ^{18}F-fluorodeoxyglucose positron emission tomography/computed tomography.

Table 5

Definition criteria of *C burnetii* focalized infection.

(Part I) Definition of *C burnetii* osteoarticular infection (without prosthesis)	(Part II) Definition of *C burnetii* lymphadenitis
Definite criterion	Definite criterion
Positive culture, PCR or immunochemistry of bone or synovial biopsy, joint aspirate	Positive culture, PCR, immunohistochemistry, or fluorescence in situ hybridization of lymphadenitis
Major criteria	Major criteria
Microbiology:	Microbiology:
Positive culture or positive PCR of the blood	Positive culture or positive PCR of the blood
Positive serology with IgGI antibodies ≥800	Positive serology with IgGI antibodies ≥800
Evidence of bone or joint involvement:	Evidence of lymph node involvement:
Clinical arthritis, osteitis, or tenosynovitis	Clinical lymphadenitis
CT scan or ultrasonography (for joint) or MRI: osteo-articular destruction, joint effusion, intra-articular collection, spondylodiscitis, synovitis, acromio-clavicular localization	CT scan or ultrasonography (for joint) or MRI: lymphadenitis >1 cm
PET scan or indium leukocyte scan showing a specific osteo-articular uptake	PET scan showing a specific lymph node uptake
Minor criteria	Minor criteria
Serological IgGI ≥400 <800 mg/dL	Serological IgGI 400 <800 mg/dL
Fever, temperature ≥38°C	Fever, temperature ≥38°C
Mono or polyarthralgia	
Diagnosis definite	Diagnosis definite
1A criterion	1A criterion
2B criteria	2B criteria
1B criterion and 3C criteria (including 1 microbiological characteristic)	1B criterion and 2C criteria (including 1 microbiological characteristic)
Possible diagnosis	Possible diagnosis
1B criterion and 2C criteria	1B criterion and 1C criteria
3C criteria	2C criteria

I = definition of *C burnetii* osteoarticular infection (without prosthesis), II = definition of *C burnetii* lymphadenitis, *C burnetii* = *Coxiella burnetti*.

vascular foci involved a Bentall graft, so that these infections were systematically considered to be associated with prosthetic endocarditis, and 2 cases showed an associated hypermetabolism on a native valve. This shows that vascular *C burnetii* infections cover 2 different entities: primary infection of a pre-existing aneurysm or vascular graft (which seems to be the more frequent) and real "mycotic aneurysm" as a consequence of Q fever endocarditis. Historically, the definition of "mycotic aneurysm" was provided by Osler in 1885, with the description of a "mushroom-shaped" aneurysm secondary to infectious endocarditis embolism in the arterial wall.[36] These aneurysms are more frequently saccular. Thus, we think that the term "mycotic aneurysm" that has been used generically in several studies dealing with Q fever vascular infections[37] should be used only in the case of associated endocarditis, that is, in cases of valvulopathy associated with a vascular aneurysm in a context of Q fever infection. ^{18}F-FDG PET/CT, which provides a systemic view of infected foci, is a key tool in the distinction of these 2 clinical entities. Some hypermetabolic foci (prostatic, thyroid, and laryngeal) remain of unknown significance, so further studies are required to monitor these foci carefully to understand their meaning and specificity. Our study is 1 more argument for the use of 18-F-FDG PET/CT in the diagnosis of infectious diseases, as recommended by the European regulatory agency,[38] because it allows to change the diagnosis in *C burnetii* infection for 62% of cases upon discovering or confirming a focus of infection.

Because Q fever is a systemic infectious disease that can affect several organs at once, ^{18}F-FDG PET/CT imaging emerges as a revolutionary tool for localizing all foci of *C burnetii* infection. Moreover, our work is a new step in demonstrating that the notion of "chronic Q fever" is inadequate because it artificially combines significantly different persistent foci of infection. ^{18}F-FDG PET/CT helps achieve a more accurate identification of infected foci. For each of the foci described, we propose a sampling strategy to confirm the diagnosis of *C burnetii* infection, which can be made—thanks to several methods such as PCR, culture, immunohistochemistry, and FISH. All these new tools will encourage the development of specific prevention and treatment strategies for each type of *C burnetii* persistent focalized infection.

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Article 7 : Treatment and Prophylactic Strategy for *Coxiella burnetii* Infection of Aneurysms and Vascular Grafts: A Retrospective Cohort Study.

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Treatment and Prophylactic Strategy for *Coxiella burnetii* Infection of Aneurysms and Vascular Grafts

A Retrospective Cohort Study

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Abstract: *Coxiella burnetii* vascular infections continue to be very severe diseases and no guidelines exist about their prevention. In terms of treatment, the benefit of the surgical removal of infected tissues has been suggested by 1 retrospective study.

We present a case of a *C burnetii* abdominal aortic graft infection for which we observed a dramatic clinical and biological recovery after surgery. We thus performed a retrospective cohort study to evaluate the impact of surgery on survival and serological outcome for patients with Q fever vascular infections diagnosed in our center.

Between 1986 and February 2015, 100 patients were diagnosed with Q fever vascular infections. The incidence of these infections has significantly increased over the past 5 years, in comparison with the mean annual incidence over the preceding 22 years (8.83 cases per year versus 3.14 cases per year, $P = 0.001$). A two-and-a-half-year follow-up was available for 66 patients, of whom 18.2% died. We observed 6.5% of deaths in the group of patients who were operated upon at 2 and a half years, in comparison with 28.6% in the group which were not operated upon ($P = 0.02$). Surgery was the only factor that had a positive impact on survival at 2 and a half years using univariate analysis [hazard ratio: 0.17 [95% CI]: [0.039–0.79]; $P = 0.024$]. Surgery was also associated with a good serological outcome (74.1% vs 57.1% of patients, $P = 0.03$). In the group of patients with vascular graft infections (n = 47), surgery had a positive impact on serological outcome at 2 and a half years (85.7% vs 42.9%, $P < 0.001$) [hazard ratio: 0.40 [95% CI]: [0.17–098]; $P = 0.046$] and tended to be associated with lower although not statistically significant mortality (11.1% vs 27.6% of deaths, $P = 0.19$).

Surgical treatment confers a benefit in terms of survival following *C burnetii* vascular infections. However, given the high mortality of these infections and their rising incidence, we propose a strategy that consists of screening for vascular graft and aneurysms in the context of primary Q fever, to decide when to start prophylactic treatment, similar to the strategy recommended for the prophylaxis of Q fever endocarditis.

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Abbreviations: 18 FDG PET/CT = 18 F-Fluorodeoxyglucose positron emission tomography/computed tomography, *C burnetii* = *Coxiella burnetii*.

INTRODUCTION

Q fever is a zoonosis caused by *Coxiella burnetii*. The primo infection can take several clinical forms such as pneumonia, influenza-like illness, and hepatitis. When the infection persists, it can lead to Q fever endocarditis in patients with valvulopathy and also to vascular infections.[1] The main known risk factors for vascular infections are aneurysms and the presence of vascular grafts.[2] Diagnosis of vascular infections can be made using several criteria, including high levels of Phase I IgG titers to *C burnetii* (see Table, Supplemental Digital Content 1, http://links.lww.com/MD/A835),[1] and new imaging tools such as 18 FDG PET/CT have been proposed recently to help detect these infections.[3–5] A comprehensive literature review, including recent studies from the outbreak in the Netherlands, yielded a total of 230 reported cases of *C burnetii* vascular infections,[5–27] including 90 cases of vascular graft infections. The majority of the reports were published in the last 10 years, suggesting an increase in clinicians' awareness of these infections in association with the development of 18 FDG PET/CT in this indication.

These infections have a very poor outcome because of a major risk of death resulting from aneurysm, graft tear or fistulization to adjacent organs.[6,10,28] Overall mortality varies between 18% and 25%.[6,17] However, very few studies are available regarding the influence of surgical removal of the infected vascular tissue on prognosis. Some reports describe surgical treatment of these infections with successful outcomes[8,20,21] and 1 study suggests that surgery confers a survival benefit[6] when combined with the recommended antibiotics, doxycycline, and hydroxychloroquine. Conversely, a recent retrospective study from the Netherlands suggests that surgical treatment of chronic Q fever is associated with all-cause and chronic Q fever-mortality.[17] These results seem difficult to interpret, since in the classification used by the authors, endocarditis and vascular *C burnetii* infections are grouped under the global term of ''chronic Q fever.'' However, patients with vascular graft infections often suffer from multiple vascular comorbidities that increase the anesthesia and surgical risks of these interventions, making the surgical decision difficult.

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Moreover, no large prospective study has been found in the published literature dealing with the treatment and prognosis of *C burnetii* vascular infections, because of the number of cases per center being too small.

A successful prevention strategy exists for Q fever endocarditis. As these infections occur in patients with a pre-existing valvulopathy or prosthetic valve, we propose a strategy to systematically search for a valvulopathy (using transthoracic echocardiogram) in patients with primary Q fever,[29] and to initiate prolonged prophylactic treatment with doxycycline and hydroxychloroquine in such patients. This approach has resulted in a dramatic decrease in the incidence of endocarditis over a 6-year period.[30] In infections of vascular aneurysms or prosthesis, a screening strategy to decide when to perform prophylactic treatment would be useful.

We describe the case of a patient who had a *C burnetii* infection of his aortic graft. Local surgeons contraindicated the operation and he presented poor evolution following antibiotic treatment alone. When he was finally operated on abroad, we observed a dramatic serologic decrease after surgery, testifying to its positive evolution. We retrospectively analyzed the incidence and characteristics of patients with Q fever vascular infections over a 29-year period in the French National Referral Center for Q fever. We also retrospectively assessed the role of surgery in *C burnetii* aneurysms and vascular graft infections on the survival and serological outcomes for these patients.

Case Presentation

A 34-year-old Lebanese patient was transferred from the hospital in Beirut to the Timone Hospital in Marseilles, France, on January 23, 2008 for fever and polyarthralgia. His medical history included aortic prosthesis surgery in March 2001, due to a chronic traumatic aortic rupture. The CT scan revealed an eso-aneurysmal fistula with an associated aortic collection and a renal abscess. Q fever serology was performed in our laboratory and was found to be positive (IgG phase I: 6400 and IgG phase II: 12,800). Our patient had 2B criteria, that is definite diagnosis of Q fever vascular prosthesis infection according to the *C burnetii* vascular infection score (see Table, Supplemental Digital Content 1, http://links.lww.com/MD/A835).[1] As recommended in our center, treatment with doxycycline and hydroxychloroquine was initiated. We put this patient forward for surgical treatment. All surgeons refused to operate on him, considering the surgical risks to be too high. Twelve months later, he suffered from several deep abscesses (renal, splenic, and pulmonary) and bacteremia. Only 1 dilution decrease of IgG phase I was noted, suggesting a poor outcome. At 18 months, a new fistula on the prosthesis appeared with an endoluminal vegetation. On our advice (DR), the patient contacted a surgeon (HS) practicing in Liverpool, United Kingdom, who decided to perform surgery. He was finally operated on there for surgical debridement with graft replacement and fistulous tract repair. Antibiotherapy with doxycycline and hydroxychloroquine was continued. *C burnetii* was detected by qPCR on the graft biopsy. After the intervention, we observed a dramatic clinical improvement and a 5-fold decrease in Q fever serological titers 4 months later. (IgG phase I titer = 100 and IgG phase II titer = 200) (Figure 1). Doxycycline and hydroxychloroquine were continued for 16 months, that is 3 and a half years in total. At the time of publication, 4 years after the surgery, the patient is in good health, with no clinical or serological relapses.

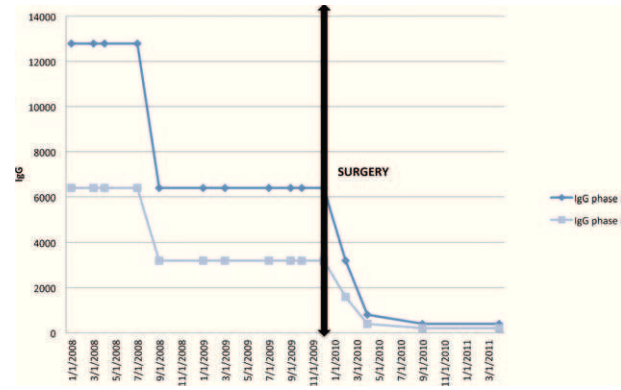

FIGURE 1. Serological and clinical outcome of the case patient before and after surgery.

Patients and Methods

Patients

We screened the French National Referral Center for Q fever database for patients with Q fever vascular infections between 1986 and February 2015. Over this period, we identified 100 patients who had the diagnosis criteria for vascular infections. Vascular infection was defined using published proposed criteria for diagnosis of Q fever vascular infection (see Table, Supplemental Digital Content 1, http://links.lww.com/MD/A835).[1] Patients were considered to have a vascular infection when *C burnetii* was isolated by culture or molecular detection in a vascular aneurysm or vascular graft biopsy. Major criteria were IgG titer to phase I *C burnetii* >6400 or molecular detection from the patient's blood combined with a CT scan or 18 FDG PET/CT showing vascular involvement (see Table, Supplemental Digital Content 1, http://links.lww.com/MD/A835).

For each patient, we retrospectively collected their sociodemographic data (age and sex), clinical data (type and location of vascular prosthesis or aneurysm), and serological data. We also noted the presence of surgical treatment with removal of the infected tissue or device in addition to the recommended antibiotherapy, combining doxycycline, and hydroxychloroquine. Vital status at 1, 2 and a half, and 3 years of follow-up was sought by phone call when patients were not clinically followed in our center. Serological outcome at 1, 2 and a half, and 3 years was recorded in our center's database for serologic tests for Q fever.

Methods for Diagnosing *C burnetii* Vascular Infection

Serological diagnosis was performed in our laboratory using an indirect immunofluorescence assay to determine IgG titers to phases I and II of *C burnetii,* as previously described [6]. Molecular detection of *C burnetii* was performed on blood or surgical samples of vascular tissue or graft when available. DNA was extracted using the QiAmp tissue kit (Qiegen, Hilden, Germany) and detection of *C burnetii* was performed by qPCR targeting the IS1111 repetitive element.[31] For culture, samples were inoculated HEL cells as previously described.[32]

Outcome Criteria

Good serological outcome was defined by a 2-fold decrease in the dilution titer of IgG antibody to *C burnetii*

phase I, or a decrease of IgG I antibody below 800. Survival outcome was recorded by collecting dates of death for deceased patients, and the last time they were seen in consultation or by direct phone call to their medical doctor at 1, 2 and a half, and 3 years of follow-up. The outcome of interest was the elapsed survival time from diagnosis to either death or the end of the follow-up period. We chose to perform the final statistical analyses with a follow-up time of 2 and a half years because this was when data were available for the highest proportion of patients, providing the best statistical power.

Statistical Analysis

A bilateral Barnard exact test was used to test associations in 2×2 tables.[33] This test was preferred to the classical exact Fisher test because it is more powerful for small samples.[34] A P value <0.05 was considered to be significant. Comparison of means was performed using a Student t test. The impact of surgery on survival and serological outcome at 2 and a half years was identified initially by using the Kaplan–Meier estimate and log-rank test, and secondly using Cox proportional hazards regression. We computed univariate regressions and also considered age, sex, presence of a vascular prosthesis, and the prosthesis type as a possible confounder in a multivariate analysis. Statistical analysis was performed using SPSS Software 22 and SMP software for the Barnard test.

Ethics Statement

Patients' medical data were retrospectively reviewed, and all collected data were anonymized in standardized forms according to procedures of the Commission Nationale de l'Informatique et des Libertés (the French commission for data protection). The study was approved by the local ethics committee (Comité de Protection des Personnes Sud Mediterranée 1) under registration number 1355. All patients gave informed consent.

RESULTS

From January 1, 1986 to February 2015, 100 patients were diagnosed with *C burnetii* vascular infection in our laboratory. The mean annual incidence of vascular infection in the last 5 years has increased when compared with the mean annual incidence of the preceding 22 years (8.83 cases per year vs 3.14 cases per year, $P = 0.001$) (Figure 2). In the same period, a total of 4691 cases of acute Q fever and 943 cases of endocarditis were diagnosed and we observed a 10-fold increase in the ratio of vascular infections to acute Q fever between 1986 and 2014 (Figure 2).

Clinical data and treatment at the time of diagnosis were available for 86 patients. At diagnosis, 39 patients (45.3%) had IgGI titers at 6400 or higher. The median value of IgGI titer was 3200 and the mean value was 11,926 due to extreme high values (3 patients had IgGI > 100,000). Of these 86 patients, 32 of 35 patients (91%) from whom a surgical sample was available had a positive qPCR for *C burnetii* on surgical vascular biopsy or perivascular collection. For 14 of these 32 patients (43%), the qPCR was positive after initiation of an adapted antibiotherapy and the average duration of treatment before positive qPCR on biopsy was 7.6 months (See Figure, Supplemental Digital Content 2, http://links.lww.com/MD/A835). Of these patients, 28% (2 patients with vascular graft and 2 patients without vascular graft) had a positive qPCR 1 year after the start of treatment (Figure 2). Fourteen of 24 patients (58%) had a positive culture from surgical samples (see Figure, Supplemental Digital Content 2, http://links.lww.com/MD/A835), and 9 of them (64%) had a positive culture once antibiotherapy had begun. The average duration of treatment before positive culture on biopsy was 2.3 months and 1 patient presented a positive culture after 1 year of treatment (see Figure, Supplemental Digital Content 2, http://links.lww.com/MD/A835). All patients but 1 with positive culture had a positive qPCR. Only 8 (14%) out of 55 patients had a positive qPCR on blood samples.

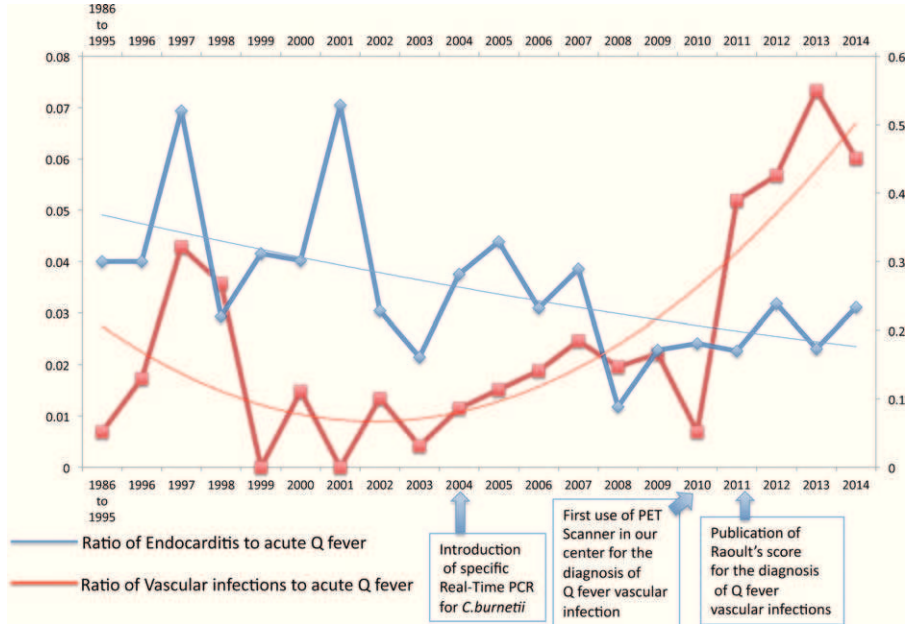

FIGURE 2. Comparison of the ratio of endocarditis to acute Q fever cases versus the ratio of vascular infection to acute Q fever cases diagnosed from 1986 to 2014.

TABLE 1. Characteristics of Patients Diagnosed With *C burnetii* Vascular Infections

	Vascular Graft	No Vascular Graft	Total	*P*
Mean age	64.7	65.8	65.09	0.6
Male sex	53 (91.4%)	26 (92.9%)	79 (91.9%)	1
Type of graft				
Aortic	51 (87.9%)	/		
Nonaortic	7 (12.1%)	/		
Surgery	20 (34.5%)	20 (71.4%)	40 (46.5%)	0.002
Total	58 (67.5%)	28 (32.5%)	86	

Twenty-two patients (25%) benefited from an 18 FDG PET/CT showing signs of vascular involvement (see Figure, Supplemental Digital Content 3, http://links.lww.com/MD/A835). For 15 patients, there was a hyperfixation of the vascular graft and for 7 patients a hyperfixation of the aneurysm wall. For 5 patients, a hyperfixation of a psoas abscess complicating the vascular infection was seen. Three patients had associated spondylodiscitis visualized on an 18 FDG PET/CT and 1 patient had an aorto-digestive fistula.

A total of 58 patients (67.5%) had a vascular graft infection and 28 (32.5%) had a vascular infection without graft. The mean age of patients was 65, and there was no difference in age between patients with or without a prosthesis (Table 1). 91.9% of patients (n = 79) were men. Forty-six percent (n = 40) of patients had a surgical treatment for their Q fever vascular infection. Patients with vascular graft infections were significantly less frequently operated on than patients with vascular aneurysm infections (34.4% vs 71.4%, $P = 0.002$). The majority of grafts involved the aorta (87.9%) (Table 1).

Impact of Surgery on Survival Status

Survival status at 2 and a half years of follow-up was available for 66 patients. The overall mortality at 2 and a half years was 18.2% (n = 12 patients). Of the patients who were operated upon, 93.5% (n = 29) were alive at 2 and a half years vs 71.4% (n = 25) in the nonoperated group ($P = 0.02$). The mortality in the subgroup of patients without vascular graft who were not operated on was 33.3% (n = 2) in comparison with no patients in the operated group ($P = 0.04$) (Table 2). The same trend was observed in patients with a vascular graft between patients who had been operated upon and those who had not, although this was not statistically significant (11.1 % vs 27.6% respectively, $P = 0.2$) (Table 2). To seek whether surgical treatment had an impact on prognosis, a Kaplan–Meier estimator was performed (Figure 3). Surgery had a significant positive impact on survival at 2 and a half years of follow-up ($P = 0.022$) (Figure 3). Then the Kaplan–Meier estimator was stratified for the presence or absence of a vascular graft. In patients without a vascular graft, this impact was statistically significant ($P = 0.029$) and for patients with a vascular graft, the Kaplan–Meier survival analysis was not statistically significant ($P = 0.19$) (see Figure, Supplemental Digital Content 4, http://links.lww.com/MD/A835).

When univariate Cox proportional hazard regression analysis was performed on the entire cohort, age, sex, presence of vascular graft, and localization of vascular graft had no influence on survival at 2 and half years. Surgical treatment was the only factor significantly associated with survival at 2 and a half years [hazard ratio: 0.17 [95% CI]: [0.039–0.79]; $P = 0.024$].

Impact of Surgery on Serological Outcome

We analyzed the serological outcome at 2 and a half years and stratified the analysis on the presence or absence of a graft. These data were available for 55 patients. 74.1% (n = 20) of patients who were operated upon had a good serological outcome at 2 and a half years, vs 57.1% (n = 16) of patients who were not operated upon ($P = 0.03$). Regarding the group of patients with vascular graft infections, 85.7% (n = 12) of operated patients had a good serological outcome at 2 and a half years of follow-up, versus 42.9% (n = 9) of patients who were not operated upon ($P < 0.001$) (Table 2). In this group, a univariate Cox proportional hazard regression analysis for each factor that could be associated with a good serological outcome at 2 and a half years was performed. Surgery was a predictor of good serological outcome at 2 and a half years of follow-up [hazard ratio: 0.40 [95% CI]: [0.17–098]; $P = 0.046$]. No other factor had a significant impact on serological outcome in this group. In the group of patients with no vascular graft, no significant difference was observed between patients who had been operated on and those who had not in terms of serological outcome at 2 and a half years (Table 2).

DISCUSSION

We report here a large detailed retrospective cohort of *C burnetii* vascular infections. Over the last 5 years, we have observed a significant increase in the annual incidence of vascular infections diagnosed in our center compared with the preceding 22 years (8.83 cases per year vs 3.14 cases per year, $P = 0.001$) (Figure 2). Botelho et al had already noted an increase in the number of diagnosed cases of *C burnetii* vascular infections between 1998 and 2006, due to a strategy to systematically screen for Q fever patients with aneurysms or vascular prosthesis and whose serum, blood, or vascular biopsies were sent to our laboratory. The recent elaboration of a diagnosis

TABLE 2. Comparison of the Vital Status and the Serological Outcome Between Patients Who Were Operated Upon and Patients Who Were Not

	Surgery (%)	No Surgery (%)	Total (%)	*P*
Death at 2.5 yr	2 (6.5)	10 (28.6)	12 (18.2)	0.02
Vascular graft	2 (11.1)	8 (27.6)	10 (21.3)	0.2
No vascular graft	0	2 (33.3)	2 (10.5)	0.04
Good serological outcome at 2.5 yr	20 (74.1)	16 (57.1)	36 (65.5)	0.03
Vascular graft	12 (85.7)	9 (42.9)	21 (60)	<0.001
No vascular graft	8 (61.5)	7 (100)	15 (75)	0.06

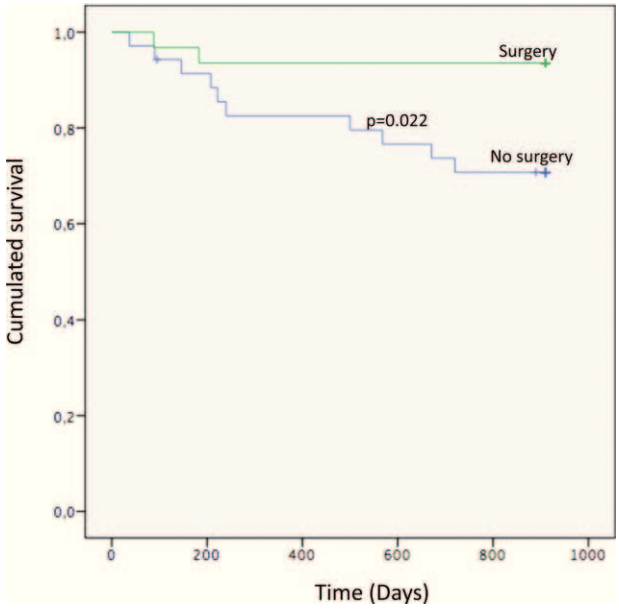

FIGURE 3. Kaplan–Meier survival curve analysis at 2 and a half years between patients who were operated upon and those who were not.

score[1] and the new contribution of 18 FDG PET/CT may also have contributed to the better identification of cases[3] (Figure 2). The mean age (65) and sex ratio (91.9% of men) in our study are fairly similar to those reported in previous studies.[6,8]

Our results confirm that *C burnetii* vascular infections are very severe with an overall mortality rate of 18.2% at 2 and a half years of follow-up. These results contrast with what we observed for Q fever endocarditis (Figure 2). Thanks to well-established management strategies, Q fever endocarditis is now a much less severe infection, with reported overall mortality rates of 7% at 3 years of follow-up.[35] Over the same period, as the result of adopting a strategy to detect and systematically administer prophylaxis to patients with risk factors for endocarditis, we have observed a dramatic reduction in *C burnetii* endocarditis incidence.[30] This is illustrated by the ratio of endocarditis to acute Q fever, which has decreased over time (Figure 2). Currently, no systematic strategy exists for detecting predisposing vascular aneurysm or vascular prosthesis in the context of primary Q fever, so no prophylaxis can be initiated to decrease the incidence of vascular infections. However, given their severity, it would seem urgent to elaborate a screening strategy for early diagnosis and prophylaxis. We therefore propose such a strategy (Figure 4). The presence of a vascular graft should be systematically looked for in the medical history of patients diagnosed with a Q fever primo infection. If a vascular graft is present and no other criteria for vascular infection are found (Figure 4), prophylaxis consisting of a 12-month treatment of hydroxychloroquine and doxycycline should be administered. Major risk factors for aortic aneurysms are: men over the age of 65 who smoke or have smoked, and a family history of aneurysms.[36] We suggest that patients over the age of 65 who are diagnosed with a Q fever primo infection undergo a CT scan or abdominal ultrasound (if renal contraindication) to screen for the presence of an aortic abdominal aneurysm. If an aneurysm is detected, prophylaxis should also be given. Eighteen FDG PET/CT could be helpful in the presence of an aneurysm or vascular graft, to detect precocious signs of infection, particularly in the event of persistent fever or poor serologic evolution (Figure 4).

Because our study is retrospective, we obtained available data on survival at 2 and a half years for only 66 patients, which has reduced the statistical power of the exercise and which constitutes a limitation of this work. In particular, in patients with vascular grafts we observed a lower proportion of deceased patients in the group of patients who had been operated upon (11% vs 27%), but the difference was not statistically significant probably because the sample of patients was too small (n = 2 and 8 respectively).

Our study represents the largest cohort of patients with Q fever vascular infection (n = 22) who benefited from an 18 FDG PET/CT. For 9 of these patients (40%), this examination revealed complications of the infection such as psoas abscesses, spondylodiscitis, or digestive fistula, reflecting the usefulness of this tool in the initial evaluation of Q fever vascular infections. Globally, *C burnetii* vascular infections remain underdiagnosed because of a lack of awareness among clinicians leading to a considerable delay in treatment. In the Netherlands, a country that faced a significant Q fever outbreak between 2007 and 2010, recent work in 1 center over a 3-year period reports a seroprevalence of Q fever of 13.6 % among 149 patients with a vascular graft, of whom 25% (5 patients) had a serologic profile compatible with a vascular infection.[7] Such studies in other European countries would be useful to better estimate the global prevalence of the disease to propose accurate treatment.

We found a positive impact of surgery on survival. These results back up those of a previous study, which showed the same link between surgical treatment and recovery.[6] This surgical benefit is significant in the subgroup of patients without a vascular graft, where we observed no deaths in the group which had been operated upon (see Figure, Supplemental Digital Content 4, http://links.lww.com/MD/A835).

For 43% of patients with a positive qPCR on vascular biopsy (n = 14), the biopsy was performed after initiating antibiotherapy (see Figure, Supplemental Digital Content 2, http://links.lww.com/MD/A835) and for 3 patients (21%) the qPCR was positive after 2 years of treatment, with 1 patient also positive in culture after this delay. This result may illustrate the fact that in *C burnetii* vascular infections, the levels of bacteria are so high that even a long course of adapted antibiotherapy is insufficient to completely sterilize the focus of infection. This possibly explains why surgical removal of the infected tissues seems to be mandatory to completely eradicate the infection. This is another difference with respect to Q fever endocarditis, for which a recent study has demonstrated that an adapted medical treatment with doxycycline and hydroxychloroquine for 18 months in native valve endocarditis and 24 months for prosthetic valve endocarditis is sufficient to eradicate the infection.[35]

The aim of our study was also to check whether the positive impact of surgery on prognosis could be seen in the particularly high-risk population of patients with vascular graft infections (such as the case of our patient). In these patients, we found that surgical removal of the infected device was a predictor of good serologic outcome, which in turn is an indicator of recovery, exactly as we observed for our patient shortly after surgery (Figure 1). Although we saw a lower percentage of deaths in patients with vascular graft infections when they were operated upon, the comparison was not

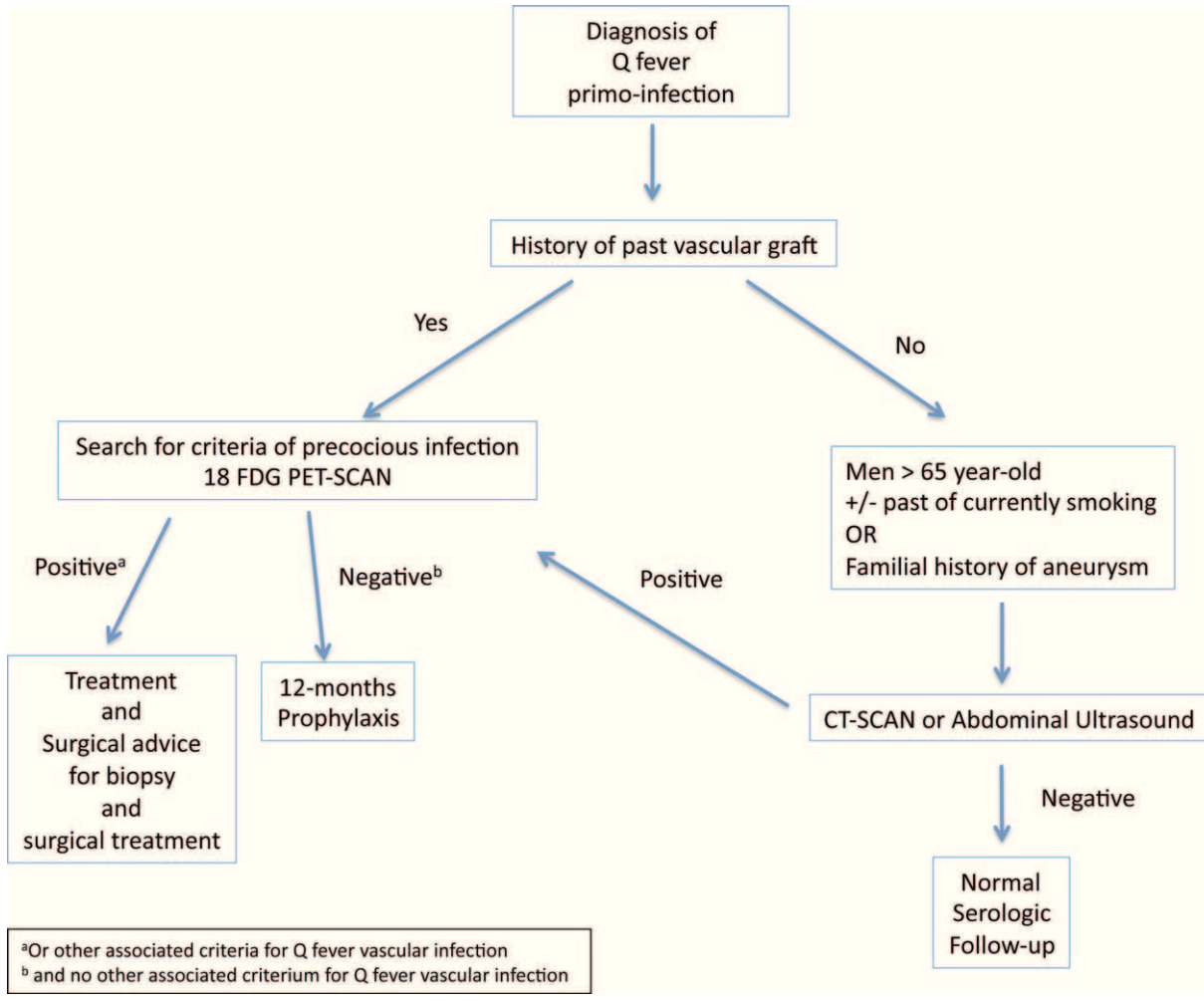

FIGURE 4. Screening strategy for vascular Q fever infection.

statistically significant. This is due to the fact that in the group which was operated upon, 2 patients died very shortly after the surgery (within 2 days), of immediate postoperative complications. Of the 2 patients, 1 succumbed on the day of his operation for graft removal from hemorrhagic shock and the other died from a myocardial infarction 2 days after surgery. Patient one, who died during the operation, was 80-year old and had a medical history comprising myocardial infarction, carotid endarterectomy, and an ischemic cerebral stroke 1 year previously. Patient two was 67-year old and died 2 days after surgery from a myocardial infarction. The coronarography revealed tritroncular lesions. His medical history was marked by a 22-year period of tobacco smoking and dyslipidemia. Currently, cardiac complications are responsible for 42% of noncardiac surgery perioperative mortality.[37] More specifically, aortic and major vascular surgery are considered high-risk procedures with more than a 5% global risk of cardiovascular death in the 30 days following surgery without considering the patient's comorbidities.[37] Within the population of patients with vascular graft infections, the assessment of cardiac risk depends on the patient's characteristics and on the type of surgery and emergency.[37] Recent predictive models to assess global surgical risk have been built and 3 of them are recommended by the American and European College of Cardiology's updated guidelines.[38,37] We applied 1 of these models, the universal ACS NSQIP surgical risk calculator[39] (www.riskcalculator.facs.org), to the patient who died from myocardial infraction. This patient had a perioperative risk of cardiac arrest or myocardial infarction of 21%. Such new tools should be applied to patients with *C burnetii* vascular graft infections to better assist the therapeutic decision. Recently, Kloppenburg et al[40] reported a case of *C burnetii* aortic bifurcated stent graft infection in a patient with high-risk comorbidities. He was treated with semiconservative surgery, with removal of the abscesses and aneurysm wall, but conservation of the endograft. Such a strategy may be a solution for patients presenting a high surgical risk.

Our study confirms that surgical treatment has a positive impact on survival in *C burnetii* vascular infections. In the subgroup of patients with vascular graft infections, this surgical treatment has no significant impact on survival, but a positive impact on serologic evolution. Because these infections remain very severe and the surgical risk continues to be very high, we propose, for the first time, a strategy involving systematic screening of vascular graft and aneurysms and prophylaxis for patients diagnosed with a Q fever primo infection. We hope that such a strategy will help to decrease the incidence and mortality of *C burnetii* vascular infections in the future.

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Partie V : Conclusions et Perspectives

Les avancées récentes dans la compréhension de l'infection à *Coxiella burnetii* ces dernières années, ont fait tomber un certain nombre de paradigmes. Sur le plan de la culture de la bactérie, l'élaboration d'un milieu de culture axénique est une étape majeure. La transformation génétique est en effet beaucoup plus aisée grâce à ce milieu de culture avec des délais de 16 jours pour obtenir des souches transformées contre 8 à 12 semaines auparavant sur culture cellulaire. Notre travail représente une étape de plus dans l'optimisation des conditions de culture de *C. burnetii*. Celles-ci peuvent néanmoins encore être optimisées avec pour objectif pratique de cultiver *C. burnetii* à partir de prélèvements cliniques en routine. Une autre étape à accomplir est la réalisation d'antibiogrammes sur milieu de culture axénique. Cependant l'une des contraintes probables sera que ce milieu a un pH acide ce qui a pour particularité de diminuer l'efficacité de certains antibiotiques et notamment de la doxycycline. Ceci représente donc un défi majeur à venir.

Le séquençage de plusieurs souches de *C. burnetii* a permis de mieux comprendre les déterminants de la virulence de cette bactérie. La revue de la littérature que nous avons réalisée sur la génomique dans l'étude de *C. burnetii* a permis de constater que la sévérité de la primo infection semble liée principalement à des facteurs génétiques de la bactérie. En revanche, toutes les tentatives essayant de relier des caractéristiques génétiques à une forme « chronique » d'infection ont été vaines. Notamment il semble ne pas exister de corrélation entre le type de plasmide présent dans une souche et la persistance ou non de l'infection. Au contraire, il apparaît au vu des données actuelles de la littérature que l'évolution vers un foyer infectieux persistant dépend principalement de facteurs d'hôtes. Cependant les facteurs influençant l'apparition de syndrome de fatigue chronique post infectieux ou de lymphome restent encore à élucider.

Notre travail a permis également de décrire et de mieux comprendre l'épidémiologie des infections à *C. burnetii* à Cayenne en Guyane Française. En effet la présence d'un génotype

unique caractérisé par une réduction de génome importante et spécifique de cette zone géographique a probablement un lien avec la virulence et la prévalence de l'infection à Cayenne. Néanmoins pour l'heure, les conséquences phénotypiques de cette délétion restent à élucider. L'utilisation de modèles animaux en ce sens pourrait être utile. Par ailleurs, le rôle du système de sécrétion de type 1 qui est concerné par cette délétion est pour le moment inconnu chez *C. burnetii*. On sait que chez *Legionella pneumophila* celui-ci joue un rôle lors de l'internalisation dans la cellule hôte. Le rôle de ce système chez *C. burnetii* nécessite d'être investigué par une approche physiopathologique.

Concernant les manifestations cliniques des infections à *C. burnetii*, une transition a eu lieu entre la dichotomie rigide fièvre Q « aigue » /fièvre Q « chronique » et la situation actuelle où une myriade de tableaux cliniques est en cours de description. Notamment le dogme selon lequel des immunoglobulines de phase I élevées étaient synonymes d'infection chronique a été battu en brèche par l'exemple des primo infections à Cayenne. A l'inverse, le terme endocardite n'est plus synonyme de chronicité puisque des endocardites aigue à *C. burnetii* ont décrites. Par ailleurs, les nouveaux critères diagnostiques que nous avons proposé pour les infections ostéoarticulaires et ganglionnaires dans lesquels le TEP scanner occupe une place majeure, vont probablement permettre d'améliorer la détection précoce de ces infections et de les étudier sur des cohortes de patients plus importantes. Cela ouvre également la voie à des essais thérapeutiques basés sur des critères diagnostiques mieux codifiés. Sur le plan thérapeutique, Le traitement de la primo infection ainsi que celui des endocardites sont déjà bien codifiés. Nous avons par notre travail confirmé le bénéfice de la chirurgie chez les patients atteints d'infections vasculaires. Cependant, l'enjeu majeur reste la prévention de ces infections par la détection précoce des facteurs de risque et un traitement prolongé par doxycycline et hydroxychloroquine quand cela est le cas, comme ce qui est fait pour la prévention des endocardites à *C. burnetii*. Il faudra évaluer à distance si cette stratégie porte ces fruits. Enfin,

de nouvelles stratégies thérapeutiques avec des traitements plus courts et ayant un meilleur profil de tolérance devraient être évalués. Des études randomisées comparant de nouveaux protocoles aux anciennes approches thérapeutiques qui ont été utilisées depuis 20 ans seront nécessaires et sont probablement l'un des principaux sujets de recherche à venir.

ANNEXES

AVANT-PROPOS

Durant ce travail de thèse, j'ai également participé à deux travaux annexes.

Le premier est un article de type « viewpoints » publié dans Plos Negected Tropical Diseases, concernant l'épidémiologie particulière de la fièvre Q en Guyane Française, et s'interrogeant sur la présence de la bactérie dans le reste du continent Sud Américain où la prévalence de la maladie est très peu décrite à ce jour. Des investigations dans ces pays sont nécessaires afin d'évaluer le réel impact de l'infection qui reste aujourd'hui négligée dans ces régions.

Le deuxième article est un commentaire concernant une étude néerlandaise sur le syndrome de fatigue chronique post primo infection à *C. burnetii.* Nous avons voulu souligner la nécessité d'une nouvelle nosologie concernant *C. burnetii* qui prendrait mieux en compte l'histoire naturelle de la maladie qui peut se résumer comme suit. La primo infection à *C. burnetii* peut-être symptomatique ou non, ce qui dépend à la fois de la souche impliquée et des facteurs de risque de l'hôte. Indépendamment de la sévérité de cette primo infection, les complications à long terme peuvent être les suivantes : infections focalisées persistantes, syndrome de fatigue chronique, ou lymphome dans certains cas, qui sont déterminés principalement par les facteurs de risques présents chez l'hôte.

Article 8 : Q fever in French Guiana : Tip of the Iceberg or Epidemiological Exception ?

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PLOS | NEGLECTED TROPICAL DISEASES

VIEWPOINTS

Q Fever in French Guiana: Tip of the Iceberg or Epidemiological Exception?

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The Distribution of Knowledge and Neglect

Q fever is a cosmopolitan zoonosis caused by an intracellular bacterium, *Coxiella burnetii*. Since its discovery in 1935 in Australia, its presence has been reported almost worldwide in animals and humans [1]. In most developed countries, this infection has been widely described, and its life cycle, exposure factors, and clinical and biological pictures are well known. The incidence of Q fever is generally quite low, and most of the cases are diagnosed during short outbreaks related to direct or indirect contact of humans with cattle, sheep, or goats, which are the main reservoirs. In developing countries, information on endemicity is generally scarce and limited to seroprevalence studies in exposed populations or case reports. This presumably reflects misdiagnosis, rather than lower incidence. The diagnosis of acute Q fever mostly relies on the elevation of anti-*C. burnetii* antibodies by 15 to 21 days after the onset of the symptoms, detected by Immunofluorescence Assay, which is the gold standard for *C. burnetii* detection. However, these diagnostic techniques are often not available in tropical areas and, apparently, in numerous Latin American settings.

Indeed, an exhaustive review of the literature in English, French, Spanish, and Portuguese showed that publications on Q fever in Latin America are scarce despite the worldwide presence of the disease (Table 1). Seven countries have never reported any cases of Q fever according to the available literature (Belize, Costa Rica, Guatemala, Guyana, Honduras, Paraguay, Suriname); three haven't reported any since 1990, but some older studies do exist (Bolivia, Panama, Venezuela); seven countries reported one or two publications since 1990 (Argentina, Chile, Ecuador, El Salvador, Peru, Trinidad, Uruguay); and Colombia, Mexico, and Brazil published several publications, including mostly case reports of chronic Q fever, one case of acute Q fever, several seroprevalence studies in exposed populations, and some studies based on an acute febrile or acute respiratory syndrome approach. Recently, Q fever was confirmed in

Table 1. Review of the English, Portuguese, Spanish, and French scientific literature (using the terms "Q fever" and "*Coxiella burnetii*" in MEDLINE and Google) among Q fever in South and Central America (except the Caribbean) since 1990, except French Guiana.

Country	Year of publication[1,2]	Type of study	Number of cases	Context
Argentina	2000 [2]	Retrospective descriptive study	1	One case among 408 hospitalized pneumonias in Buenos Aires
Brazil	2006 [3]	Cases series	16	Investigation among 726 febrile illness in Minas Gerais 2001–2004
Brazil	2006 [4]	Retrospective descriptive study	1	Investigation among 61 blood culture–negative endocarditis, Cardiology Hospital, São Paulo
Brazil	2008 [5]	Seroprevalence study	4/125 (3.2%)	Seroprevalence among HIV patients in Rio de Janeiro
Brazil	2008, 2011, 2012 [6–8]	Case report	3	One endocarditis in São Paulo, one chronic fever PCR positive, and one pneumonia in Rio de Janeiro
Brazil	2013 [9]	One case into a large prospective study on infective endocarditis	1	One PCR positive on surgical endocarditis, Rio de Janeiro
Brazil	2015 [10]	Longitudinal observational study	4	Study among dengue-suspected cases in Rio de Janeiro state, four Q fever cases confirmed by PCR and sequencing
Chile	2003 [11]	Seroprevalence study	36/116 (31%)	Agricultural and Livestock personal
Colombia	2006 [12]	Seroprevalence study	19/81 (23.6%)	Livestock farming individuals living in towns within Cordoba and Sucre departments
Colombia	2012, 2014 [13,14]	Case report	2	One endocarditis and one asymptomatic case in a rural man
Ecuador	2009 [15]	Longitudinal observational study	15/304	Study among acute febrile illness in the Ecuadorean Amazon Basin
El Salvador	1996 [16]	Seroprevalence study	18/40 (45%)	International study on three continents in Humans and animals
Mexico	2012 [17]	Cross-sectional pilot study	17	State of Hidalgo, rural area of central Mexico. Eight cases with clinical criteria
Mexico	1997, 2012, and 2013 [18–20]	Case reports	3	Granulomatous hepatitis
Peru	2004 [21]	Retrospective descriptive study	12/152 (9%)	Outbreak of febrile illness in 2002 in the district of Sapillica
Trinidad	2011 [22]	Seroprevalence study	20/455 (4.4%)	Livestock and abattoir workers
Uruguay	1994 [23]	Case report	1	Endocarditis

[1] Existing publications before 1990, but none since then: Bolivia, Panama, Uruguay, Venezuela

[2] No publication found at all: Belize, Chile, Guyana, Honduras, Suriname

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patients and animals in parts of the Brazilian Atlantic Forest (Table 1). Thus, there are no publications on Q fever in the Amazon region except in French Guiana and Ecuador.

Q Fever in Travellers and Migrants Returning from Latin America

Q fever is a rare disease in travellers, especially those returning from Latin America. Although Suriname reported no cases, one case of myocarditis due to *C. burnetii* was diagnosed in the Netherlands in an 8-year-old child whose father had recently returned from Suriname [24]. Furthermore, a seroprevalence study in the same country showed that *C. burnetii* antibodies positivity was associated with being from Suriname, Turkey, or Morocco [25]. A case of Q fever was reported in Spain in a traveller returning from 15 days of travel in the Dominican Republic and Venezuela [26]. Several cases of *C. burnetii* pneumonia were reported in travellers returning from French Guiana [27]. Recently, the French National Centre for Rickettsiosis in Marseille described genotypes of Q fever according to the presumed infection area. No case was reported in patients returning from South America, except for French Guiana.

The Singular Epidemiology of Q Fever in French Guiana

French Guiana is a French overseas territory located on the northeastern coast of South America. About 90% of its 84,000 km^2 surface is covered by the Amazonian rainforest; the remaining 10%, located in the north, consists of a coastal plain where 90% of the 250,000 inhabitants live. Almost half of the population lives in Cayenne. It is an outermost region of the European Union, with technical and financial resources that are closer to European countries than to the neighbouring countries in the fields of health and research.

C. burnetii was first described in 1955 in French Guiana, but the real interest arose in 1998 when three severe cases were described [28]. Antibodies to *C. burnetii* were tested among 275 stored samples from patients tested for dengue fever from 1992 to 1996: 9.1% were positive with a sharp increase in 1996 (23.9%). The seroprevalence was much higher in Cayenne than in rural areas. Subsequent studies found an annual incidence of 37 cases/100,000 persons between 1996–2000, up to 150 cases/100,000 persons in 2005 [29], and 17.5/100,000 persons between 2008 and 2011 [30]. *C. burnetii* primary infection is also more frequently symptomatic, with more patients presenting with fever in Cayenne compared to Metropolitan France (97% versus 81% in Marseille, $p < 0.0001$) [30]. While pneumonias only represent 8% to 37% of symptomatic Q fever in France [30], they account for about 90% of the cases in French Guiana [29,30]. While *C. burnetii* is the causal pathogen for about 1% of cases of community-acquired pneumonia requiring hospitalization in the United Kingdom and continental Europe, 2.3% in North America, and 5.8% in Israel, a highly endemic region [31], it is implicated in 24% to 38% of pneumonias in the area of Cayenne [32], which is the highest prevalence ever described worldwide. Consequently, the empirical antibiotherapy for community-acquired pneumonia in Cayenne is comprised of doxycycline in order to treat *C.burnetii*. Also, the initial presentation of *C.burnetii* pneumonia in Cayenne is severe, with more frequent symptoms like chills, headache, night sweats, and arthromyalgia than pneumonias from other aetiologies [32]. This high rate of symptomatic *C. burnetii* primary infection has a significant public health impact. Regarding persistent focalized infections, the incidence of *C. burnetii* endocarditis is the same in Cayenne as in Metropolitan France [30], and further studies are needed to assess the prevalence of endocarditis and vascular infections by *C. burnetii*, which are very severe diseases that are probably underestimated in this territory. The strategy of screening for risk factors for endocarditis (valulopathy and valvular prosthesis) by systematic echocardiography is the same as the one recommended in Metropolitan France. If a risk factor is detected, a prophylactic treatment (doxycycline and hydroxychloroquine) should be initiated because it has proven its efficacy in reducing the incidence of such infections [33].

C. burnetii epidemiology in French Guiana remains unclear: groups at risk are not clearly defined, and the classical risk factors are not observed, especially professional exposure to cattle. The main risk factors for *C. burnetii* infection are working in construction/public works, living near bats, wild mammals, or the forest, levelling work, and gardening [29]. Surprisingly, French expatriates were more frequently infected than people from other communities in French Guiana. The hypothesized reservoir remains currently controversial. Several studies have tested bats, cattle, sheep, goats, small mammals, domestic mammals, and birds, in vain [29,34]. Recently, the three-toed sloth (*Bradypus tridactylus*) has been incriminated as a possible reservoir of the bacterium in Cayenne. *C. burnetii* MST 17 has been detected in the spleen, stools, and ticks of a dead sloth near a recent outbreak site [34]. In addition, Q fever incidence was correlated with three-toed sloth birth numbers 1–2 months before, peaking during the rainy season in French Guiana [35]. However, for many animal species in French Guiana reproduction is related to the rainy season.

Although the role of the three-toed sloth in transmission is an interesting hypothesis to explore, it is probably not the only reservoir and seems unlikely to be the sole explanation for the magnitude of this problem in French Guiana. Another particularity of Q fever in French Guiana is that all the cases identified with Polymerase Chain Reaction (PCR) were due to the genotype MST 17 [36], isolated specifically from eight patients having travelled to or lived in Cayenne. Conversely, it was not detected in any of the 298 strains of *C. burnetii* from other geographical areas [36]. This unique MST 17 clone provokes an exceptional, strong immune response with very high levels of phase I IgG in the acute phase of the disease [30]. It is also more virulent, as illustrated by the high prevalence of Q fever pneumonia in French Guiana and the more severe initial presentation than pneumonias of other aetiologies [32,37]. Recently, an MST 17 strain (*C. burnetii* 175) was sequenced and revealed a unique feature: a 6105 bp-deletion in the *hlyCABD* operon of the Type 1 Secretion System (T1SS). This deletion has been detected by qPCR in eight other MST 17 strains and in none of the 298 strains of the French National Referral Centre database [38]. The genome reduction observed in the MST 17 clone is possibly linked to its exceptional pathogenicity and emergence in Cayenne.

Local Emergence or Widespread Neglect?

Q fever is supposed to be well known and cosmopolitan. Nevertheless, the contrast between the high incidence and prevalence among pneumonias in French Guiana and the near absence of data in neighbouring countries is intriguing. It may be simply due to circumscribed emergence. However, this raises the question of the underdiagnosis of *C. burnetii* infections due to lack of diagnostic tests and the lack of awareness by physicians in the Amazonian region, where no cases were reported. This infection should be found in surrounding countries, as infectious agents are not contained by borders. Several cases of acute Q fever are diagnosed in Europe in travellers returning from the countries of the Amazon, and only endocarditis and severe cases are published in the Brazilian medical literature (Table 1). Thus, these cases may be considered as the tip of the iceberg. Although at this point estimates are speculative, the potential incidence of Q fever in French Guiana could be 17.5 to 150/100,000 inhabitants per year. Based on this estimate and assuming similar incidence in countries with similar fauna in the Guiana Shield (Guyana, Suriname, French Guiana, and Amapá combined have approximately 2,230,000 inhabitants), there may be 440 to 3,330 undiagnosed cases per year. Expanding this to the Amazonian region, including northern regions of Brazil (Acre, Rondônia, Para, Roraima, Amazonas, and Tocantins combined have approximately 17,423,343 inhabitants), estimated cases might be 2,960 to 26,135 cases a year. These computations of the potential burden of Q fever are estimates with incomplete data and don't include populations of the Amazonian areas of Colombia, Venezuela, Ecuador, Bolivia, and Peru.

It is difficult to believe that *C. burnetii* would limit its spread beyond the borders of French Guiana. This apparent "emergence" in the territory with the highest GDP per capita of the South American continent, thus with the highest diagnostic resources, suggests that a plausible explanation of the gap of cases of Q fever in most of the Amazonian part of South America is one of a vicious cycle in which a lack of diagnostic tools leads to lack of evidence from diagnostic algorithms, perpetuating the lack of diagnostic tools. It is nevertheless possible that other countries in the Amazon region do not have a high incidence of Q fever. Indeed, Nova Scotia in the 1980s had very high rates of Q fever [39], but these rates were never seen elsewhere in Canada. Ultimately, studies need to be done to test this point.

The many singularities of Q fever in French Guiana warrant further studies throughout the Amazon, such as prospective studies among fevers of unknown origin, with a special focus on community-acquired pneumonia, and molecular studies on wild animal reservoirs and

transmission. Better diagnostic techniques and rapid diagnostic tests, routine PCR, better surveillance systems, and intensified international collaboration are needed to map the true burden of Q fever in Latin America. This knowledge would then help to adapt treatment protocols of pneumonia and avoid the chronic consequences of Q fever that may develop when adequate treatment is not given. These investigations will help to propose adapted screening, prophylaxis, and treatment strategies for Q fever in this region.

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Article 9 : Moving form Q fever to *C. burnetii* infection

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Moving from Q fever to *C. burnetii* infection

To the Editor

This paper by Van Loenhout *et al.* [1] gives us the opportunity to discuss the accuracy of the EU case definition for notification of Q fever, and more generally the current nomenclature of this infection.

In the EU definition, cases of 'acute Q fever' are defined by the combination of clinical symptoms (fever, pneumonia, hepatitis) and laboratory criteria. As stated by the authors, the major limitation of this classification is that it probably significantly underestimated the number of cases of *Coxiella burnetii* primary infection during The Netherlands epidemic. Interestingly, the use of this definition can be used to compare symptomatic (notified as 'acute Q fever') and asymptomatic or poorly symptomatic (non-notified) *C. burnetii* primary infections. The main significant differences between these two groups are age and sex, with symptomatic patients being significantly older and more often men than asymptomatic or poorly symptomatic patients. This result confirms what has already been described in previous studies: age and sex are the two major determinants of clinical manifestations of *C. burnetii* primary infection, with older men being more frequently symptomatic than young women, pregnant women and children [2–4]. Moreover, the results of Van Loenhout *et al.* confirm that there is no correlation between clinical manifestations of *C. burnetii* primary infection and the long-term consequences of the infection, with no significant difference in long-term health status between notified and non-notified patients [5].

These elements bring new arguments for an updated nosography of *C. burnetii* infection. We consider that the natural history of *C. burnetii* infection is quite close to what is observed in *Mycobacterium tuberculosis* infection (TB), so that the old simplistic classification of 'acute' and 'chronic Q fever' is no longer accurate. In TB, the primary infection can be symptomatic or not. If left untreated, and in the presence of host factors like immunosuppression or age, long-term complications of the infection can occur and affect different organs (Pott's disease, meningitis, miliary, lymphadenitis). Localization of the focus of infection is necessary to determine treatment duration and prognosis. In the case of *C. burnetii*, the primary infection can be symptomatic (currently described as 'acute Q fever') or not, depending on two major determinants: the strain involved [6] and the patient's susceptibilities (age, sex, pregnancy, immunosuppression) [7]. Then, long-term complications can be classified into two main entities: persistent focalized infections and fatigue syndrome (without an identified focus of infection). These complications are not linked to the severity of the primary infection but mainly to host factors. There is no 'chronic Q fever' with multiplying *C. burnetii* without a focus of infection and different focalized persistent infections have different risk factors, prognoses and treatments. *C. burnetii* endocarditis occurs in subjects with pre-existing valvulopathy, and is associated with high IgG anticardiolipin antibody titres during primary infection [8]. Its prognosis has improved thanks to prophylaxis, early diagnosis and treatment [9]. Vascular infections occur in patients with pre-existing aneurysm or vascular grafts, requires surgical treatment and still has a very poor prognosis [10]. Conversely, no death has been reported to date in patients with *C. burnetii* osteoarticular infections [11, 12]. *C.burnetii* infection during pregnancy is frequently asymptomatic but can lead to severe obstetrical complications like fetal death and malformations [13]. Finally, we recently demonstrated that *C. burnetii* persistent lymphadenitis can lead to lymphoma [14].

Regarding fatigue syndrome, a disease without evidence of *C. burnetii* multiplication and not treatable with antibiotics, this paper demonstrates that it is equally frequent after *C. burnetii* symptomatic and asymptomatic primary infections. Recently, another study from The Netherlands on 'Q fever fatigue syndrome' used the definition of the National Dutch consensus guidelines, which is cited as follows: 'a sudden onset of fatigue related to a symptomatic acute Q fever infection' [15]. Given the data from Van Loenhout *et al*. [1], this definition is obviously misleading. Further studies using a definition allowing detection of fatigue syndrome after *C. burnetii* asymptomatic primary infections are necessary to reach a conclusion on the actual occurrence and risk factors of this syndrome.

In conclusion, the work of Van Loenhout *et al.* stresses the need for moving from the old unspecific 'Q fever' nomenclature to more precise definitions of the different forms of *C. burnetii* infection in order to establish efficient public health strategies. In epidemic situations like the one experienced in The Netherlands, detection and follow-up of asymptomatic *C. burnetii* primary infections should be a priority, especially in high-risk patients (valvulopathy, vascular aneurysms or prosthesis, osteoarticular prosthesis, pregnant women) to detect and prevent the development of both focalized persistent infections and fatigue syndrome.

Declaration of Interest

None.

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