Coxiella burnetii and the diagnosis of Q fever

Coxiella burnetii is the infectious agent responsible for Q fever, which occurs worldwide [1]. Many reservoirs have been reported, including mammals, birds and arthropods (mainly ticks), but infectious aerosols produced by farm animals and pets, including those from feces, milk, hides and wool, are the most frequent source of human infection [1]. Person-to-person transmission is rare [1,2], although sexual transmission has been documented [3].

Presentation of the disease is extremely variable. A non-immunized person develops a primary infection in 60% of cases (Table 1). This can lead to the acute disease (in 40% of cases), which mostly presents as a flu-like syndrome or as severe pneumonia; 2% of patients with acute disease are hospitalized [1]. In patients with pre-existing valvulopathy, infection can progress to the chronic form (in 2-5% of patients), which is characterized by bloodstream-negative endocarditis [1,4]. The fever and characteristic vegetations (a mixture of bacteria and blood clots on heart valves) are frequently absent, making diagnosis difficult [1]. Importantly, Q fever is associated with high morbidity and mortality in pregnant women [1,4], although only few such cases have been reported to date [2,4]. The incidence of Q fever was recently re-evaluated by analyzing Q fever data collected at the French National Reference Center (FNRC) between 1985 and 2009 [5]. During this 25-year period, the FNRC identified 32 outbreaks in Europe, indicating that the number of Q fever cases was increasing [5].

In the recent outbreak of Q fever in the Netherlands, a rapid increase in human Q fever cases (3,523 in total) was observed between 2007 (182) and 2009 (2,361) [2,6,7]. Q fever had already been endemic in the Netherlands, but at the disease was previously diagnosed in dairy goats and dairy sheep in 2005 [2,7]. The sudden increase may have been linked to a more virulent subtype of C. burnetii [2,6,7]. Indeed, several genotypes of C. burnetii were involved in the Dutch outbreak. When tested by multiple-locus variable-number tandem repeat analysis (MLVA) typing, the strains were found to differ by only a single repeat difference and it was thought that they might represent microvariants of a hypervirulent strain [7]. The rising number of reported outbreaks over the past 10 years worldwide is, however, considered to be a consequence of more efficient detection [6]. In the Dutch outbreak, several factors were considered to have contributed to the increase in Q fever cases, including: (i) the high density of farms in the regions where the bacterium is endemic, (ii) asymptomatic infection in the majority of infected animals, and (iii) more efficient diagnostic tests [2,6,7]. Nevertheless, important factors still need to be assessed including the persistence of C. burnetii in the environment and in different hosts, and the potential to prevent and control the next outbreak. Q fever has
become a serious public health problem in many areas not previously known as endemic zones. The bacterium is highly infectious and, consequently, the Centers for Disease Control and Prevention (CDC) in the USA have classified it as a category B bioterrorism agent [8].

In the past decade, technological developments have contributed an improved understanding of some of the pathological aspects of the intracellular life-cycle of \( \text{C. burnetii} \) and the role of host immunity. The diagnosis of \( \text{C. burnetii} \) infection still lacks sensitivity and specificity, especially at the early stage of infection. Therefore, recent efforts have focused on identifying strain-specific or clinical-outcome-specific protein markers (Table 2). Here, we review recent studies on \( \text{C. burnetii} \), focusing on the contribution of proteomic technologies to our understanding of \( \text{C. burnetii} \) infection and to the diagnosis of Q fever.

**Coxiella burnetii**

**Bacteriology**

\( \text{C. burnetii} \) is a small (0.3–1 µm) obligate intracellular Gram-negative coccobacillus. The cell wall structure of this bacterium, which displays characteristics similar to those of Gram-negative bacteria, but does not stain reliably with Gram stain; for this reason, Gimenez staining has been used historically [9]. \( \text{C. burnetii} \) has been classified as a member of the γ-proteobacteria [1,8].

**Genetic variability of isolates**

Currently, the genome sequences of six \( \text{C. burnetii} \) strains (CBuG Q212, CBuK Q154, Dugway 5J108-111, RSA331, RSA493 and MSU Goat Q177) are available; Nine Mile RSA493 was the first \( \text{C. burnetii} \) genome to be sequenced [10,11]. \( \text{C. burnetii} \) isolates also harbor different plasmid types (QpH1, QpRS, QpDG or QpDV), which define specific genovars [8]. It remains to be determined whether these plasmids are involved in virulence.

Voth et al. [12] suggested that \( \text{C. burnetii} \) plasmids play an important role in host-cell modifications [12]. Proteins encoded by plasmid QpH1 (such as CBUA0014) are translocated into the host cell by the Dot/Icm type IV secretion system (T4SS). Compared to those of other strictly intracellular bacteria, the \( \text{C. burnetii} \) genome harbors multiple copies of insertion sequence (IS) elements that are probably involved in genomic plasticity [13], but it possesses fewer pseudogenes, suggesting a recent genome reduction event [10]. The T4SS, together with genes encoding a large proportion of basic proteins, including ion exchangers that enable the bacterium to live in an acidic environment, are characteristic of the \( \text{C. burnetii} \) genome [10].

**Phase variation**

In addition to the reported genetic variability, antigenic variation due to different lipopolysaccharide (LPS) structures [3,14] and sugar compositions [15] is common among \( \text{C. burnetii} \) isolates. This is frequently referred to as phase variation. Smooth, full-length LPS is characteristic of isolates from naturally infected biological samples (phase I, virulent), whereas rough, truncated LPS is found in sub-cultured bacteria (phase II, avirulent). Unique \( \text{C. burnetii} \) carbohydrates have been identified and studied in detail [14,16]. Among these are 3-C(hydroxymethyl)-1-lyxofuranose, known as dihydroxy-streptose (Strep), and 6-deoxy-3-methyl-D-gulopyranose, known as virenose (Vir), a unique marker of phase I virulent strains [14]. Recently, the virulent phase I and avirulent phase II variants of the Nine Mile RSA493 and RSA439 strains of \( \text{C. burnetii} \) were compared using tandem liquid chromatography mass spectrometry.

### Table 1. Main characteristics of the immune responses to \( \text{C. burnetii} \) infection occurring in the acute and chronic phases

| Immune response | Acute phase | Chronic phase | Detection or exploration methods |
|-----------------|-------------|---------------|----------------------------------|
| Cells           | T lymphocytes | Fewer T lymphocytes (CD4/CD8) | Flow cytometry |
| Total eradication of bacteria | No | No | qRT-PCR, qPCR |
| Granuloma formation | Yes | No, large vacuole | Immunohistochemistry |
| Detection of bacteria in granuloma/large vacuole | No, very weak | Yes | Immunochemistry and qRT-PCR, qPCR |
| Antibody production | IgG against phase I, IgM against phase II | IgG, IgM, IgA against both phase I and II | Serology (IFA) |
| Properties of monocytes from convalescent patients | Able to kill \( \text{C. burnetii} \) and migrate through the endothelium | Unable to kill \( \text{C. burnetii} \) or migrate through the endothelium | qRT-PCR, qPCR targeting \( \text{C. burnetii} \), apoptosis detection (TUNEL assay) |
| Cytokines | IFN-γ and TNFα, mediated through TLR4 activation | IL-10 | qRT-PCR targeting the specific cytokines |

IFA, immunofluorescence assay; IFN-γ, Interferon-gamma; IgG, Immunoglobulin G; IgM, Immunoglobulin M; IgA, Immunoglobulin A; IL-10, Interleukin 10; qRT-PCR, quantitative real time polymerase chain reaction; qPCR, quantitative polymerase chain reaction; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
Table 2. Main protein candidates for serodiagnosis that have been cross-validated by proteomic studies

| Protein (locus-tag) | Identification | MW (kDa) | pI | Protein function | Nature of protein | Peptide signal sequence | Nature of analyzed samples | DT | Clinical significance | Ref(s) |
|-------------------|----------------|----------|----|------------------|------------------|------------------------|--------------------------|----|----------------------|--------|
| CBU_0952          | Acute disease antigen A (adaA) | 25.9     | 8.67 | Unknown          | Membrane         | MKKLTVTLFLH           | Cb isolates              | BT, IP | Marker of acute Q fever | [31,63] |
| CBU_0612          | Putative outer membrane chaperone protein (ompH, Skp) | 18.8     | 9.71 | Molecular chaperone, interacts with unfolded proteins | Membrane*        | M1KRLS1AICS-            | Patient sera, Cb NM II TPE | IP, RP | Marker of Q fever endocarditis, SP with Q fever patients (general) | [56,57] |
| CBU_0937          | Hypothetical protein | 51.4     | 8.99 | Unknown          | Membrane         | MTSKVSALGL CVSGALSTT LAST | mAbs, Cb NM II TPE, RP-based ELISA/HS | IP, BT | Marker of Q fever endocarditis, Marker of phase II SP with both acute Q fever and Q fever endocarditis. Marker of both phase I and phase II | [17,56,58,65,67-69] |
| CBU_1910          | Outer membrane protein (com1) | 27.6     | 9.08 | Protein disulfide oxidoreductase, unknown role in pathogenesis | Membrane         | M1NRLTALF LAGTLTAGIAA APSQF | mAbs, Cb NM II TPE | IP, BT, RP | Marker of chronic Q fever | [17,58,65,67-69] |
| CBU_0236          | Elongation factor Tu (tuf-2) | 43.5     | 5.32 | GTP-dependent binding of aminoaclyl-tRNA in protein biosynthesis | Soluble**         | M1Abs, TPE Cb NM II, HS, AS (infected/vaccinated guinea pigs) | IP | SP, marker of acute Q fever | [31,58,65,68] |
| CBU_0092          | Tol-pal system protein (YbgF) | 34.3     | 6.46 | Critical for maintaining integrity of bacterial outer membrane Involved in protein-protein interactions | Membrane         | M1R1IKM1K1TLC VSSALAAALM LSAPL1WADA | TPE Cb NM I and II HS Q-fever (general), AS (immunized guinea pigs) protein microarray | IP, RP | Phase II-specific marker (early diagnosis of acute Q fever), marker of Q fever (general) | [68,69] |
| CBU_0311          | Outer membrane protein (Coxiella burnetii P1) | 26.8     | 8.44 | Able to form pore in lipid bilayers | Membrane*        | OM location shown for Cb NM I | METTTRLAI0V/ ALCCLASAA FAGGD | RP, IP | Marker of Q fever (general), marker of acute Q fever; applications for drug and vaccine development | [31,67] |
| CBU_1718          | Chaperonin (GroL) | 58.284   | 5.14 | Protein folding, ATP hydrolysis | Soluble'         | HS/ TPE Cb NM II / RP; IP HS, and AS (infected/vaccinated guinea pigs) | IP, RP | Marker of Q fever (general), marker of acute Q fever | [31,65] |
| CBU_0229          | 50S ribosomal protein L7/L12 (RplJ) | 13.2     | 4.71 | Binding site for several factors in protein synthesis | Membrane'        | MAQLSKDDI LEAVANMSV MDWDLVK AMEEKFGVS AQAIAAVAG PVAGGAEA | IP; HS, and AS (infected/vaccinated guinea pigs) | BT, IP | Marker of both phase I and phase II, marker of acute Q fever | [17,31] |
| CBU_0263          | DNA-directed RNA polymerase subunit alpha (rpoA) | 35.5     | 5.61 | DNA-dependent RNA polymerase transcription | Soluble          | OMP fraction of Cb NM II and CbuG_ Q212 II; Phase 1 HS (chronic) | IP | Marker of chronic Q fever | [2,69] |
| CBU_1916          | Universal stress protein family | 15.78    | 6.58 | Stress response | Soluble*         | OMP fraction of Cb NM II and CbuG_ Q212 II; Phase 1 HS (chronic) | IP | Marker of chronic Q fever | [1,69] |

L, phase I; II, phase II; AS, animal sera; BT, biotyping; Ch, Coxiella burnetii; DT, discovery technology; HS, human sera; IP, immunoproteomics; mAb, monoclonal antibody; MW, molecular weight; NM, Nine Mile; OM, outer membrane; OMP, outer membrane protein; pI, isoelectric point; RP, recombinant protein-based approaches; SP, seroreactive proteins; TPE, total protein extract.

Soluble (cytosolic). Membrane (having a signal peptide that directs protein to the cell membrane). *More than two-fold more abundant in the LCV stage than in the SCV stage. †Proteins common to SCV and LCV.
of strain-specific and clinical-outcome-specific protein markers [17] (Table 3). A total of 235 and 215 non-redundant proteins were identified from phase I and II variants, respectively. The most interesting outcomes of this work were the identification of 17 proteins that are involved in LPS biosynthesis, the first identification of DotD protein of the T4SS, and finally the identification of two ankyrins (CBU_0898 and CBU_1482). Biomarkers of LPS phase I were identified and might contribute to development of more sensitive diagnostic tests [17].

**Culture conditions**

*Coxiella burnetii* is cultured in level 3 biosafety laboratory conditions. The bacterium can be propagated under laboratory conditions in cell lines [18] or in embryonated eggs [19, 20]. *C. burnetii* is able to infect various types of cells, including monocyte-macrophage systems and macrophage, fibroblast and epithelial cells [3,21]. The isolation of bacteria from clinical samples is carried out using the shell vial centrifugation technique [1]. *C. burnetii* was recently described as being cultivable in axenic medium (a medium that is free of contaminating organisms) under laboratory conditions [22]. The bacteria can be grown when incubated in a mesophilic atmosphere in an acidified citrate medium that is enriched with cysteine and casamino acids [22] and contains divalent metal cations. LimB (CBU_1224a), a unique *C. burnetii* lipoprotein identified using matrix-assisted laser desorption ionization-time of flight/time of flight MS (MALDI-TOF/TOF MS), serves as surface receptor for such ions and may be involved in *C. burnetii* replication and pathogenesis [23]. Notably, the *C. burnetii* proteome includes a eukaryotic-like A24 sterol reductase homolog, CBU_1206, which might be involved in the intracellular growth of the bacterium [24].

**Host-bacteria interactions**

**Physiopathology**

Immune control of *C. burnetii* infection depends on T lymphocytes: chronic Q fever has been shown to develop preferentially in a nude mouse model that has a greatly reduced number of T cells [8]. In acute-phase disease, granuloma formation is a hallmark of an efficient immune response (Table 1), but *C. burnetii* is frequently missing from granulomas, resulting in the inability of PCR or immunocytochemistry tests to produce a positive diagnosis. In the chronic phase of infection, the immune response is inefficient or deleterious [8] (Table 1). The inoculum size, route of infection, host factors, and pathogenic potential of strains all play a role in the clinical presentation of acute Q fever [8]. Age, circadian rhythms and sex-related differences [25] may be involved in the development of the chronic form. Female sexual hormones (17-β-estradiol) are thought to have a protective role [8,26].

**Intracellular survival**

*C. burnetii*, an obligate intracellular bacterium, has evolved not only to survive but to thrive in the phagolysosome. The intracellular survival of *C. burnetii* is characterized by two distinct morphological forms: the large cell variant (LCV), which has evolved to persist within the acidified phagolysosome of monocytes or macrophages, and the ‘spore-like’ small cell variant (SCV), which can persist both in the phagolysosome and in extreme environmental conditions [27]. How *C. burnetii* mediates the establishment of the phagolysosomal-like compartment in which it resides and replicates is not well understood. We do know that bacterial protein synthesis is required for this process, suggesting that bacterial proteins directly influence the biogenesis of the *C. burnetii*-occupied vacuole [28,29]. Some of these mechanisms have been elucidated using proteomics and molecular biology [28-31]. Both developmental forms (SCV and LCV) were analyzed by a combination of two-dimensional electrophoresis (2-DE) and MS after differential fractionation [31]. Fifty proteins were identified *in vitro* from cytoplasm from Vero cells that were infected with *C. burnetii* (Table 3), but their roles have not been determined [31,32]. A Dot/Icm-dependent translocation in host cytoplasm was demonstrated for only a few of these, including *Coxiella* effector proteins such as CpeA (CBUA0006), CpeB (CBUA0013), CpeC (CBUA0014), CpeD (CBUA0015), CpeE (CBUA0016) and CpeF (CBUA0023) [29,32]. The T4SS candidate proteins identified by proteomic approaches remains to be functionally validated [31,32]. The majority of the identified proteins were found to be important for the intracellular survival of bacteria and were involved in RNA and DNA processing [33], confirming the results of Coleman *et al.* [31]. Notably, most of the identified proteins had basic physicochemical properties and contained eukaryotic motifs (such as ankyrin repeat-containing domains (Anks)) [33]. When *Legionella pneumophila* was used as a surrogate host, several different *C. burnetii* Anks could be delivered into the host cells by the *L. pneumophila* T4SS, suggesting that *C. burnetii* T4SS effector proteins affect host cell signal transduction pathways [28,34]. Moreover, when ectopically expressed, the *C. burnetii* Anks localized to a variety of subcellular regions in mammalian cells [32]. An understanding of the trafficking and role of Anks and of the secretion of T4SS effectors could help in selective drug design.

**Current approaches for diagnosis of Q fever**

The major issue for Q fever diagnosis is the non-specific clinical picture produced by the disease. Early stage
### Table 3. Proteomic approaches for *C. burnetii* biomarker selection

| Approach | Technique(s) | Cb isolates (culture method*) | Sera/mAbs | Applications | Identified proteins | Ref(s) |
|----------|--------------|-------------------------------|-----------|--------------|---------------------|--------|
| **Biotyping** | | | | | | |
| Detection of specific markers for Cb isolates | MALDI-TOF MS | Cb: RSA493, BUD, Priscilla (CYSEE) | - | Optimization of method for typing Cb for specific strains or clinical isolates | RSA493-specific: CBU_1637, CBU_0401, CBU_0394, CBU_1592, CBU_1378, CBU_0403, CBU_0078, CBU_0961, CBU_1609, CBU_0644, CBU_1055 Priscilla-specific: CBU_0149, CBU_0438, CBU_0168, CBU_0745 BUD-specific: CBU_1989, CBU_2085 | [20,54] |
| Identification of Cb strains and isolates | MALDI-TOF MS | Cb: NMI, Australian QD, M44, KAV, PAW, Henzerling, Ohio (CYSEE) | - | Cb isolate typing or diagnosis | Mass spectral peaks (1000-6000 Da), species-selective and strain-specific | [55] |
| Proteome of Cb NM I and II | 2-DE and nanoLC-ESI LC-MS/MS, MALDI-TOF/TOF MS, MALDI-TOF MS | TPE Cb RSA493, NM I (CC) | - | Markers of early stage infection, and therapeutic or vaccine development | 197 distinct proteins. | [19] |
| In silico prediction of OMPs and identification of Cb (NM II) LP | Bioinformatics | Cb RSA493 genome sequence | - | Vaccine development or serodiagnosis | 21 predicted OMPs and 9 LP, CBU_1190 (LoLA), CBU_1829 (LoLB) | [59] |
| Proteome of Cb strain NM phase II | 2-DE, 2D SDS-tricine PAGE, MALDI-TOF | TPE Cb NM II (CC, Vero) | - | Markers of active Cb infection, serology or therapeutic development | Proteins involved in Cb pathogenesis and survival mechanisms (NM II) | [94] |
| Proteome of Cb I and II | LC-MS/MS | Cb NM I and II (CYSEE) | - | Phase I and phase II distinct biomarkers, serology or biotyping (blood transfusion) | 150 proteins reported (pI > 9.5), virulence type I and T4SS: CBU_0884, CBU_0085, CBU_0318, CBU_0744, CBU_1099, CBU_1352, CBU_0338, DotD protein (CBU_1643); Anks: CBU_0898, CBU_1482; 7 enzymes involved in LPS phase I synthesis: CBU_0676, CBU_0678, CBU_0674, CBU_0681, CBU_0682, CBU_0683, CBU_0691, CBU_0846, CBU_1657 | [17] |
| **Cb secretome** | Tricine-SDS PAGE, ESI-MS/MS, bioinformatics | Cb NM II (CC, Vero) | Cytoplasmic fraction from infected Vero cells | Characterization of T4SS, drug development | 50 T4SS effector candidates: CBU_1440, CBU_0132, CBU_1001, CBU_1386, CBU_1518, Orf145, QpH1_p21, CBU_1297, CburgD_01001397 | [33] |
| Two Cb strains, subproteome (OMPs) and candidate proteins for serodiagnosis | Tricine-SDS PAGE, double SDS-PAGE, IP 2-DE, MALDI TOF/TOF | Cb strains NM RSA 493 II (acute) and CbG Q212 II (chronic), (CC, Vero) | OMP-enriched fraction, 1 HS (chronic) | Subproteome of Cb, chronic Q fever markers | 86 identified OMPs. | [56] |
| **Immunoproteomics or serology** | IP/serodiagnosis | 17 Cb strains (CC) (BGM or L929) | RP ada (rada), A5 immunized with rada | Marker of acute Q fever | adaA (CBU_0952) | [31,63] |
| Candidate proteins for acute Q fever serodiagnosis | IP (2-DE, MALDI-TOF) | Purified SCVs and LCVs | HS (convalescent-phase, acute Q fever), A5 from infected or vaccinated guinea pigs | Subunit vaccines or serodiagnostic for acute Q fever, unique SCV/LCV markers | Proteins that are differentially expressed in SCV and LCV forms. | [31] |
| Identification of Cb cell-variant-specific common SCV/LCV proteins | IP (2-DE/MALDI-TOF) | TPE Cb NM II (CC, Vero) | HS (acute Q fever or IE Q fever) | Markers of chronic Q fever (IE), serodiagnosis | CBU_0612, CBU_0480 | [57] |

Continued overleaf
Table 3. Continued

| Approach | Technique(s) | Cb isolates (culture method)* | Sera/mAbs | Applications | Identified proteins | Ref(s) |
|----------|--------------|-------------------------------|-----------|--------------|---------------------|--------|
| Identification of Cb proteins reacting with Cb mAbs | IP (2-DE/MALDI-TOF; RP-based ELISA) | TPE Cb NM II (CC, Vero) | Specific mAbs; HS acute or chronic (IE) | Markers of chronic Q fever (IE), serodiagnosis | Q fever markers (general): CBU_1910, CBU_0236; Chronic Q fever marker (IE): CBU_0937 | [58] |
| SP for early serodiagnosis of Q fever | IP (2-DE, LC-MS/MS) | TPE Cb NM I and II (CC, L929), Cb NM RSA493 I (CYSEE) | AS from immunized guinea pigs | Markers of early stage acute Q fever, serodiagnosis or vaccine development | | |

**RP-based approaches**

- **Candidate RPs for Q fever vaccine development and serodiagnosis**
  - TAP products and expression of selected SP
  - HS (acute, chronic phase vaccine)
  - Serodiagnosis or vaccine development
  - SP: CBU_0008, CBU_0381, CBU_0612, CBU_0781, CBU_1115, CBU_1143, CBU_1157, CBU_1249, CBU_1853, CBU_1869
  - Top candidates: CBU_0781 (AnkG), CBU_1115 (lipoprotein), CBU_1143 (YajC)

- **Candidate RPs for Q fever vaccine development and serodiagnosis**
  - Protein microarray
  - HS Q fever (general)
  - Markers of Q fever (general)
  - SP: CBU_0110, CBU_0891, CBU_0109, CBU_1143, CBU_0612, CBU_0009, CBU_0545, CBU_1398, CBU_0630, CBU_1513, CBU_1719, CBU_0229, CBU_0653

- **Candidate RPs for Q fever vaccine development and serodiagnosis**
  - ELISA (HS), ELISPOT (AS)
  - 11 RPs; Cb NM RSA493 I
  - HS Q-fever (IFA-positive, convalescent), HS chronic (transgenic mice, immunized with Cb NM RSA493 I)
  - Subunit vaccine development, serodiagnosis
  - SP: CBU_0110, CBU_1716, CBU_612, CBU_716, CBU_311

- **Identification of Cb SP**
  - IP (2D, LC-MS), tripartite fusion RP
  - TPE Cb I and II Henzelering strain (CC, Vero)
  - HS Q fever (general)
  - Serodiagnosis or vaccine development
  - SP: CBU_0091, CBU_0109, CBU_0395, CBU_0867, CBU_1221, CBU_1268, CBU_1718, CBU_1910
  - Six RPs (Cb II): GroEL, Com1, RecA, EF-Tu, OmpA-like and FtsZ

I, phase I; II, phase II; adaA, acute disease antigen A; AS, animal sera; Cb, *Coxiella burnetii*; IE, infective endocarditis; HS, human sera; IP, immunoproteomics; LP, lipoproteins; MAb, monoclonal antibody; MW, molecular weight; nanoLC-ESI MS/MS, nano liquid chromatography-electrospray ionization mass spectrometry; NM, Nine Mile; OM, outer membrane; OMP, outer membrane protein; RP, recombinant protein; SP, seroreactive proteins; TAP, products, transcriptionally active PCR products, TPE, total protein extract; Vir and Strep: synthesis of virenoose and streptose.

*CYSEE, cultured on yolk sacs in embryonated eggs; CC, cultured in cell lines.

Detection of *C. burnetii* lacks specificity and is not sensitive enough for diagnosis of acute Q fever [35]. Moreover, the serological profiles of acute and chronic Q fever differ [5]. In acute cases, immunoglobulin M (IgM) is produced against phase I and II variants, and patients will have immunoglobulin G (IgG) antibodies against phase II antigens. In chronic Q fever, high levels of IgG against phase I and II antigens are produced [5] and persist for months or years after the initial infection. Increased IgG and immunoglobulin A (IgA) antibodies against phase I antigens are also often indicative of chronic Q fever [36]. In the early stage of infection (<10 days), the specific antibodies remain undetectable [37]. The prevalence of auto-antibodies, including antibodies similar to those seen in cases of rheumatoid arthritis and lupus, presents another problem in Q fever diagnosis [21].

**Direct diagnosis**

The laboratory diagnosis of Q fever depends on the stage of disease (acute or chronic), which in turn determines which sample should be used for analysis: blood, cerebrospinal fluid, bone marrow, cardiac valve biopsy, vascular aneurysm or graft, bone biopsy, liver biopsy, milk, placenta, fetal specimens in cases of abortion, or cell culture supernatants [38]. The choice of technique also depends on the available laboratory capabilities and on the clinical presentation of disease.
Immunodetection
In patients with chronic Q fever who are undergoing treatment, immunodetection of *C. burnetii* in fresh tissue samples or samples after formalin fixation and paraffin embedding may be very useful [1]. Several techniques can be employed: either an immunoperoxidase technique or immunofluorescence with polyclonal or monoclonal antibodies is frequently used [1]. New diagnostic tools, including autoimmunohistochemistry [39] and immunohistochemical peroxidase-based methods, have been reported for the diagnosis of blood culture-negative endocarditis [1]. The specificity of immunodetection is strongly correlated to the quality of the antibodies used.

Molecular tests
Several PCR-based assays have been developed in the past decade [1,37,40,41]. Although lacking sensitivity, PCR targeting the *htpAB*-associated repetitive element, which is present in 20 copies in the genome of *C. burnetii* [10], is routinely used to detect bacteria in cell cultures and clinical samples from both acute and chronic Q fever patients [1]. Light cycler nested-PCR (LCN-PCR) has been optimized for the early diagnosis of acute Q fever [40] when antibodies are absent [37]. This test, together with serology, is recommended in the first 2 weeks of acute Q fever [37]. Real-time quantitative PCR assay targeting the multicopy insertion sequences IS1111 and IS30a is also highly specific and sensitive [40,41]. Detection of the *adaA* gene (encoding acute disease antigen A) can be used to confirm acute Q fever [1]. Overall, PCR is useful for detecting *C. burnetii* in the early course of infection, following antigen shedding in livestock, or when applied to biopsies from patients with chronic Q fever. Molecular testing is generally recommended in addition to serology [2] but the possibility of reagent contamination leading to false-positive results is its major drawback [2,42,43].

Serology
The microbiological diagnosis of Q fever is usually based on serology and most commonly uses an indirect immunofluorescence assay (IFA) [2,5]. The cut-off for serological titres was first established in 1994 [44] but has been revised recently [5]. The diagnosis of Q fever is performed using different methods: a complement fixation test with commercially available antigen preparations combined with real-time PCR [45], enzyme-linked immunosorbent assay (ELISA) [46], IFA, and nested PCR [46]. ELISA helps in the diagnosis of Q fever after the fifth day of infection, whereas PCR is an efficient diagnostic tool during the first few days of infection [46].

According to guidelines for Q fever diagnosis, combined approaches, including PCR (≤7 days) and IFA (≥7 days), are strongly recommended in the early phase of infection [5,37,47] (Figure 1). In the case of chronic Q fever, especially with endocarditis, a positive result from systematic serological testing has been included as a major criterion in the modified Duke criteria [48]. When cross-reactivity with *Legionella micdadei* [49], *Bartonella* [50] and *Rickettsiae* [51] is observed, immunoblotting with adsorbed cross-reacting antigens is recommended.

The InoDiag automated fluorescence multiplexed antigen microarray method [52] has been compared with the IFA reference method for the detection of *C. burnetii* IgM. The advantages of the InoDiag technique are speed of analysis, the need for only a small quantity of sampled serum (5 μl) and multiplexing [53]. The sensitivity and specificity obtained by automated assay for diagnosis of the acute form were excellent, and the serological parameters obtained for serodiagnosis of Q fever endocarditis were also adequate [52]. This is a first step towards IFA standardization [52,53].

Proteomics
Recent technological developments in the field of molecular medicine have moved beyond genomics and transcriptomics to proteomics, with the goal of characterizing the impact of disease and therapy on cellular networks. Advances in proteomics-based research provide potential for the development of efficient diagnostic and therapeutic assays (Figure 1). Depending on the availability of clinical samples (storage, standardization and cohort), methods for proteomic analysis include mass spectrometry (MS), gel-based proteomics, 2-DE, differential gel electrophoresis (DIGE), immunoproteomics, recombinant protein-based arrays, and methods for the analysis of post-translational modifications (PTMs).

MS-based approaches
Several recent proteomics studies have been undertaken to identify clinical biomarkers that facilitate the accurate detection of the infectious agent, and offer new insights into inter- or intra-species relatedness. Several attempts have been made to characterize the whole proteome of *C. burnetii*, aiming to identify biomarkers that are useful in diagnosis or vaccine production for different strains or isolates (Table 3) [19,20,54]. These studies have helped to determine appropriate conditions for MS analysis, focusing on different matrices that can be used (such as acyano-4-hydroxyccinnamic acid is a good matrix choice for samples with molecular weight (MW) <10,000 Da; sinapinic acid is an appropriate matrix choice for samples with MW >10,000 Da; or 2,5-dihydroxybenzoic acid is a good a matrix choice for hydrophobic compounds) and the nature of the sample (such as intact bacterial cells, cell-free extracts). Altogether, these studies have improved the MS-based laboratory pipeline.
Characteristic and reproducible MS fingerprints containing unique biomarker profiles have also been obtained. This approach was applied for *C. burnetii* strain and phase identification by two independent laboratories for strains NMI, Australian QD, M44, KAV, PAV, Henzerling and Ohio [55] and for strains RSA493, BUD and Priscilla [20,54]. The method was validated by the prediction of samples in an independent test set with 100% sensitivity and specificity for five out of six strain classes [55]. Differences in the ion-signal profiles of three isolates, RSA493, BUD and Priscilla, were observed for peptides in the mass range 3-18 kDa [20,54]. In the recent work of Papadioti et al. [56], the outer-membrane protein (OMP) fractions of *C. burnetii* strains Nine Mile RSA 493 and CbuG_Q212 (phase II) were compared using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) combined with MS/MS analysis. Markers of chronic Q fever, such as CBU_0612 and CBU_0937, were identified [56-58] with agreement to predicted in silico *C. burnetii* OMPs [59].

When compared with conventional phenotypic and molecular identification methods, the implementation of MS in clinical laboratories could improve both the speed and sensitivity with which human pathogenic infections are diagnosed [60,61]. Nevertheless, proteomic approaches such as MALDI-TOF should not completely replace traditional diagnostic techniques in clinical microbiology, even though these traditional approaches have a number of shortcomings including the need for time-consuming biochemical and antibiotic sensitivity tests [60]. Recently, Hernychova et al. [62] demonstrated that *C. burnetii* can be identified rapidly at the species level by MALDI-TOF. To date, however, no routine method for the identification of *C. burnetii* clinical isolates has been shown to be fully reliable, probably because of the restrictions in culturing and handling *C. burnetii*. Further optimization of *C. burnetii* culture on solid media should facilitate its improved identification by routine biotyping.

**Immunoproteomics**

Despite the availability of sensitive and specific laboratory tests, the diagnosis of Q fever remains difficult. Moreover, a differential diagnosis to distinguish chronic (mainly endocarditis) from acute Q fever is greatly needed. Thus, several immunoproteomic studies, combining the use of combine 2-DE immunoblots and MS, have set out to find specific biomarkers of Q fever for the development of accurate diagnostic tools (Tables 2 and 3).

To date, only a few studies have investigated the possibility of differentiating between acute and chronic Q
Several markers have been proposed: (i) a marker of acute Q fever, adaA (CBU_0952) [63], and (ii) CBU_0612 (OmpH) and CBU_0480 (an arginine repressor), which were identified as promising markers for patients with Q fever endocarditis at 57. In another study, Q fever-specific proteins, namely the CBU_0937 protein, the OMP Com1 (CBU_1910) and elongation factor Tu (CBU_0236) were found to be discriminated by monoclonal antibodies [58]. Two of these proteins (CBU_0937 and CBU_1910) were cloned, expressed and tested by ELISA with sera from patients with acute and chronic Q fever [58]. Com1 (CBU_1910) has been widely studied [31,57,64-67] and is currently used for seroimmunological screening. Although tests using these immunoreactive proteins (CBU_0937 and CBU_1910) were neither sensitive nor specific enough for routine clinical application, the serological parameters for Com1 protein (CBU_1910) were cross-validated [58] and were in the same range as those reported by Beare et al. [66]. Moreover, Papadioti et al. [56] also demonstrated the seroreactivity of proteins CBU_0937 and CBU_0612 by two-dimensional immunoblot performed with serum from a patient with chronic Q fever [56].

Recent work by Deringer et al. [68] has raised the possibility of early- and late-stage serodiagnosis. These authors evaluated the IgG-specific response in a guinea pig model following vaccination with the Nine Mile strain of C. burnetii. Nine novel seroreactive C. burnetii proteins were identified (Table 3). Furthermore, several immunoreactive proteins from this study were identified in other studies as being immunoreactive with human Q fever sera [31,57,66,69]. This study did not, however, identify specific protein markers for each phase (I and II) separately. Notably, the identification of seroreactive C. burnetii proteins with low homology to other proteins seems to be promising for serodiagnosis because of the likelihood of low cross-reactivity. However, the low similarities of CBU_0937 with proteins in other bacteria were not sufficient for it to be considered useful as a specific marker. The serological operating parameters for Q fever serodiagnosis using CBU_0937 showed low sensitivity, even though the specificity was acceptable [58]. For patients with acute Q fever and endocarditis, the results were in the same range, indicating the low diagnostic potential of CBU_0937 [56,58].

Multiplexed biomarker protein patterns have a significantly higher positive predictive value (PPV) for disease discrimination. Immunoproteomic studies have been used to build a library of potential diagnostic or vaccine-related protein targets in several bacterial species: Chlamydia trachomatis [70-72], Helicobacter pylori [73-77], Francisella tularensis [78-82], Shigella flexnerii [83,84], Tropheryma whippelii [85,86] and Bartonella henselae [87,88]. Indeed, recent technological progress has enabled high-throughput, large-scale screening in miniaturized formats, such as protein microarrays. The laboratory pipeline could be enhanced by the validation of discovered diagnostic value (Figure 2). Some of the comprehensive studies performed on selected immunoproteomic targets were previously performed using molecular approaches. One such study involved H. pylori urease, which has diagnostic value (in the 13C urea breath test (UBT) and in UBT-C13/UBT-C14 urease activity-based tests) and is a vaccine candidate [74]. In addition, these immunoproteomic studies were not applied for routine diagnostics, but contributed to the selection, and in some cases validation, of specific biomarkers. Immunoproteomics is time consuming, but has been an important first step in biomarker discovery. Further progress will probably depend on the miniaturization of clinical assays and the use of recombinant proteins.

Screening of recombinant proteins

Proteomics focuses on the large-scale study of an organism's proteins, particularly their structures, functions and expression. After the identification and subsequent verification of specific protein biomarkers, their utility as highly reliable, specific diagnostic markers can be investigated using complementary methods to previously used biological tests. The combination of immunoproteomic methods with protein expression and validation techniques provides an ideal basis for this highly demanding challenge (Figure 2). The study of Chao et al. [65] is an example of the integration of complementary technologies. Eleven protein candidates were selected using an immunoproteomic approach, and six (hsp60, Com-1, RecA, EF-Tu, OmpA-like protein and FtsZ) were successfully cloned [65], but these proteins were not tested for their diagnostic potential [65]. Other studies investigated diagnostic value using serodiagnostic screening with recombinant proteins [66,67,89]. High-throughput screening for the selection of serodiagnostic candidates has recently been performed [66,69]. Transcriptionally active PCR products (TAP products) corresponding to 1,988 C. burnetii open reading frames (ORFs) were tested using a protein microarray [66]. In total, 75% of the full-length proteins were produced using an in vitro transcription and translation system, and these were screened with sera from patients with Q fever and with sera from vaccinated individuals [66]. Fifty strongly immunoreactive protein candidates were proposed as serodiagnostic markers, including several previously identified proteins [31,57,64,65,67], Ank and multiple hypothetical proteins [66]. The top ten candidates, and the most reactive hypothetical membrane-associated protein CBU_0089, are listed in Table 3 [66]. In a study from the same group [67], all 11 of these recombinant
proteins were able to differentiate a majority of IFA-positive sera from IFA-negative sera, but the reaction was stronger when sera from patients with endocarditis was used rather than sera from patients with acute Q fever. In the study by Vigil et al. [69], 84% of the entire proteome was expressed using a rapid translation system and screened with serum samples from 40 acute Q fever patients and 20 healthy individuals [69]. Only 21 antigens reacted strongly with IgG antibodies from infected C. burnetii patients [69]. Of these, 13 were specific to C. burnetii and eight cross-reacted with sera from healthy blood donors. As expected, CBU_1910 was the most reactive antigen with high specificity [69]. Among the identified proteins, several had already been identified in other studies and tested in a proof-of-principle diagnostic assay [31,57,58,66,68]. The results from Vigil et al. [69] and Beare et al. [66] showed similar ranges of reactivity for the best candidate protein biomarkers (CBU_1910, CBU_0891, CBU_1143, CBU_0612, CBU_0545, and CBU_1398).

In addition, several biomarkers were selected using immunoproteomic studies [31,57,68] and were reported to be promising proteins for Q fever serodiagnosis. In a large-scale comprehensive study, only about 1% of the
whole proteome of *C. burnetii* expressed *in vitro* showed seroreactivity [69]. This proportion of reactive antigens is comparable to that reported by Beare *et al.* [66]. Altogether, the data suggest that a limited number of proteins are involved in the humoral response to *C. burnetii* [66,69].

Recently, protein microarrays were used to evaluate the humoral response to *C. trachomatis* [90,91]. Sera from mice immunized with live and non-viable elementary bodies were screened with 99% of the genomic and plasmid proteins expressed *in vitro* [90]. The results revealed that 185 proteins elicited a strong early and sustained antibody response in mice. Indeed, most of these proteins have already been reported as seroreactive [90,91]. In similar work, 933 genomic- and plasmid-derived recombinant glutathione S-transferase (GST) fusion proteins were tested with sera from 99 women with urogenital infections. Among 27 seroreactive serum samples, 12 proteins had already been reported as having

### Table 4. Advantages and limitations of proteomic technologies in clinical microbiology

| Technical approach                  | Advantages                                                                 | Limitations                                                                                     |
|-------------------------------------|-----------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| **MS-based approaches**             |                                                                             |                                                                                                 |
| Biotyping                           | - MALDI-TOF MS has several applications in diagnosis and clinical microbiology, including the identification of bacteria and characterization of bacterial proteomes | - When several bacterial species are present (such as *Streptococcus* spp. or anaerobic Gram-negative bacteria in addition to *C. burnetii*) poor mass-peak signals may result, which may not be distinguished from the signals produced by the culture medium |
|                                    | - Selection of protein targets for diagnostic, vaccine or therapeutic development | - Limitations in *C. burnetii* handling (such as level 3 biosafety laboratory needed, difficulties of culturing an intracellular bacteria) |
|                                    | - Applicable to a variety of samples: including bacterial colonies, clinical samples such as blood cultures or urine, and environmental samples | - Blood culture is slow (with slow-growing bacteria) or negative (for example in cases of blood-culture-negative endocarditis caused by *C. burnetii*) and insufficiently sensitive (for example when the patient has previously received antibiotics) |
|                                    | - Availability of commercial database (includes 1,660 bacteria isolates and 66 cell types) |                                                                                                 |
|                                    | - Low cost and easy handling compared with classical phenotype-based bacterial identification |                                                                                                 |
| SDS-PAGE coupled to nano-LC proteome identification | - In general, better coverage and sensitivity when compared with MALDI-TOF MS | - Requires culture of the pathogen, for *C. burnetii* this involves labor-intensive purification from eukaryotic cells and processing of samples |
|                                    | - Identification of low molecular weight proteins | - Labor-intensive, time-consuming |
|                                    | - Suitable for investigations of PTMs | - Requires skilled operators |
|                                    |                                                                             | - Costly                                                                                         |
| Immunoproteomics (2-DE coupled to MALDI-TOF) | - Low cost | - Requires the culture and purification of *C. burnetii* in a level 3 biosafety laboratory, and is labor-intensive |
|                                    | - Resolved proteins contain PTMs that can be visualized on gels (isofoms) | - Requires large samples                                                                 |
|                                    | - Robust and suitable method for biomarker selection | - Variable findings can result, depending on culture conditions, strains, technology and operator skills |
|                                    | - Can provide an individual profile of reactivity for each patient sample | - Differences between 2D stained gels and immunoblots |
|                                    | - Can be used with monoclonal antibodies | - Limitations with 2-DE in resolving basic membrane proteins, and low and high MW proteins |
|                                    |                                                                             | - In general, low genome coverage (5-30% of total predicted ORFs) |
| Recombinant protein-based approaches |                                                                             |                                                                                                 |
| Protein array                       | - Does not require the culturing or handling of *C. burnetii* | - Escherichia coli system produces proteins without their PTMs (phosphorylations, glycolysations), which are known to be antigenic |
|                                    | - Miniaturized systems require small amounts of clinical samples (such as 1-2 µl sera) and allows high-throughput screening (>75% of total predicted ORFs) | - Misfolded or multimeric proteins may not be recognized |
|                                    | - Low cost, does not require specific operator skills | - Requires costly laboratory equipment (fluorescent scanner and spot robot) |
| ELISA                              | - Easy to perform, does not require sophisticated technology | - Lower sensitivity than protein array or IPCR |

IPCR, immuno-PCR; LC, liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; ORF, open-reading frame; PTM, post-translational modification.
diagnostic value and a further 15 proteins were newly identified [91]. Both studies narrowed down the number of seroreactive targets, showing that the number of proteins involved in the humoral host response is limited. A miniaturized protein microarray model has been used to investigate both the humoral response against *Burkholderia pseudomallei*, the causative agent of melioidosis (classified among the group B bioterrorism weapons by the CDC [92]), and *B. henselae*, the causative agent of cat scratch disease and infective endocarditis [93]. A few specific and sensitive antigens with diagnostic value are now available for a number of infectious diseases. The diagnostic potential of recombinant proteins might be useful in complementing the usual tests, but is insufficient to replace whole antigen-based serology of Q fever. The major drawback of recombinant proteins, generally expressed using *Escherichia coli*-based systems, is the lack of PTMs, which are of increasing interest for translational and clinical applications.

**Conclusions and future directions**

The future of diagnostic testing relies upon the development of new technologies, and proteomics is rapidly contributing to this area (Table 4). More sensitive and specific tests for early-stage Q fever detection as well as reliable methods for clinical follow-up of patients are needed.

Proteomics is paving the way for serodiagnosis development by first selecting seroreactive protein candidates and then validating them in recombinant-protein-based screening systems, such as classic ELISAs and large-scale comprehensive protein arrays. To date, however, none of the proteomics-based techniques has been applied for routine diagnosis of Q fever, mainly because the majority of the resulting discoveries are awaiting large-scale validation. Moreover, the equipment and resources available in diagnostic laboratories outside of large hospitals are generally insufficient for proteomic investigations, and the technology remains expensive and time-consuming.

One of the most important challenges in Q fever diagnosis is the detection of *C. burnetii* during the early stage of disease, because asymptomatic seroconversion is observed in only 60% of patients. Optimization of the conditions for obtaining specific MALDI-TOF signatures of *C. burnetii*-infected serum (acute Q fever) will be the first step towards the routine application of this technology. Moreover, in the event of a *C. burnetii* outbreak, MS-based approaches could be useful in strain subtyping, which in turn allows preventive measures and treatments to be used. Strain-specific proteins have been already characterized [19,20,94]. Considering that the immune response to recombinant proteins is limited, their routine use in acute Q fever diagnosis is doubtful. Nevertheless, diagnoses made using recombinant-protein-based microarrays might enhance the discriminatory power of whole antigen-based serology and PCR. This can be particularly useful when searching for serum markers of chronic infection in ‘at risk’ patients.

The specificity of Q fever serology might also be enhanced by employing monoclonal antibodies raised against *C. burnetii*, and these are available in several laboratories [58,95-101]. Such approaches can be useful for the detection of *C. burnetii* infection using serum samples and immuno-PCR (IPCR) [102,103] when no such infection has been detected by classic whole antigen-based ELISA. Routine clinical applications are still needed for the detection of intracellular pathogens. An immune-MALDI-TOF MS [104] could be another alternative for investigating chronic Q fever samples (such as biopsies of infected organs). Even in this post-genomic era, however, new technologies are not yet able to replace the isolation and culturing of pathogens. The development of *C. burnetii* axenic medium was an enormous breakthrough [22] that has allowed the genetic manipulation of these bacteria. Indeed, a *C. burnetii* genetic mutant lacking the FtsZ protein has been generated [105]. Genetic manipulation of these bacteria will allow several new areas of investigation, including further proteomic studies of the immune response to *C. burnetii*, studies of the effectors of the T4SS, and investigation of the intracellular survival mechanisms of *C. burnetii*. The information garnered in such studies will, in turn, facilitate the development of more specific and sensitive diagnostic assays for Q fever.

**Abbreviations**
ad, acute disease antigen A gene; Ank, ankyrin repeat-containing domain; CDC, Centers for Disease Control and Prevention; 2-DE, two-dimensional electrophoresis; ELISA, enzyme-linked immunosorbent assay; FNRC, French National Reference Center; IFA, immunofluorescence assay; IgG, Immunoglobulin G; IgM, Immunoglobulin M; IgA, Immunoglobulin A; IPCR, immuno-PCR; IS, insertion sequence; LCV, large cell variant; LPS, lipopolysaccharide; MS, mass spectrometry; MW, molecular weight; OMP, outer-membrane protein; ORF, open-reading frame; PTM, post-translational modification; SCV, small cell variant; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; T4SS, type IV secretion system; UBT, urea breath test.

**Competing interests**
The authors declare that they have no competing interests.

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