UNIVERSITY OF CRETE

DEPARTMENT OF CHEMISTRY

LABORATORY OF BIOCHEMISTRY

Doctoral Thesis

STUDY OF ANTIGENIC PROTEINS OF THE INTRACELLULAR PATHOGEN *COXIELLA BURNETII*, THE ETIOLOGICAL AGENT OF Q FEVER

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Supervisor: Prof. Georgios Tsiotis

HERAKLION 2020

Dedicated to my family...

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Publications

- Vranakis,I., **Mathioudaki E.**, Kokkini S., Psaroulaki A. Com1 as a Promising Protein for the Differential Diagnosis of the Two Forms of Q Fever. Pathogens. 2019 doi: 10.3390/pathogens8040242.
- Xie H., Valsamidis G., **Mathioudaki E.**, Tsiotis G.. Complete Genome Sequence of Pseudomonas sp. Strain phDV1, an Isolate Capable of Efficient Degradation of Aromatic Hydrocarbons. Microbiology Resource Announcements. 2019 doi: 10.1128/MRA.01171-18
- Psaroulaki, A*., **Mathioudaki, E**., Vranakis, I., Chochlakis, D., Yachnakis, E., Kokkini, S., Xie, H. and Tsiotis, G. In the search of potential serodiagnostic proteins to discriminate between acute and chronic Q fever in humans. Some promising outcomes.. Frontiers in Cellular and Infection Microbiology, under revision.
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Symposia, Conferences and Oral Presentations

- **Mathioudaki E.**, Vranakis I., Kokkini S., Psaroulaki A. (2019). Development of a protein-based-assay for the differential diagnosis of Q fever. 21th Chemistry Postgraduates Conference, University of Crete. May 15-17, Heraklion, Greece.
- **Mathioudaki E.**, Vranakis I., Kokkini S., Psaroulaki A (2018). Development of a protein-based-assay for the differential diagnosis of Q fever. 9th Hellenic Crystallography Association (HECRA)

International Conference University of Patras, 5-7 October 2018, Patras, Greece.

- **Mathioudaki E.**, Arvaniti K., Kourpa K., Tsiotis G., Xie H., Muenke C. (2017). Study of Secretion System Type IV of Coxiella Burnetii. 5th Chemistry Symposium in memory of Dr. Maria Chatzimarinaki, University of Crete, Department of Chemistry.
- **Mathioudaki E.**, Arvaniti K., Kourpa K., Tsiotis G., Xie H., Muenke C. (2017). Study of proteins of the Secretion System Type IV of Coxiella burnetii. Chemical Biology of Disease, Foundation for Research and Technology, September 15-18, Heraklion, Greece.
- **Mathioudaki E.**, Arvaniti K., Kourpa K., Tsiotis G., Xie H., Muenke C. (2017). Study of proteins of the Secretion System Type IV of Coxiella burnetii. 19th Chemistry Postgraduates Conference, University of Crete. May 2-4, Heraklion, Greece.
- EYCN 12th Delegates Assemply, University of Crete, May 2-4, Heraklion, Greece. Member of the organizing committee.

ABSTRACT

Q fever is a prevalent worldwide zoonosis, and has various acute and chronic clinical manifestations. *Coxiella burnetii* has been identified in a wide range of wild and domestic animals, including arthropods, birds, rodents, carnivores, ungulates and livestock. Acute Q fever is mostly a self-limiting, mild, influenza-like disease, sometimes complicated by severe pneumonia or hepatitis. A proportion of the patients infected by C. *burnetii*, will progress to persistent focalized *Coχiella burnetii* infection after primary infection. Chronic Q fever leads to high death rates if left untreated, which makes early case-finding and preventive measures critical for patients at high risk. Q fever has a high socioeconomic burden that presents significant challenges for both public and animal health. During the past 25 years, 32 outbreaks in Europe have been identified, indicating that the number of Q fever cases is constantly increasing. Q fever has become a serious public health problem in many areas not previously known as endemic zones and represents a major health risk for humans and animals. The economic and public health importance of the disease was recently underscored following the largest ever reported outbreak, which occurred from 2007 up to 2011 in the Netherlands.

Concerning the timely diagnosis of chronic Q fever, a diagnostic problem exists since the current gold standard method for the diagnosis, immunofluorescence, has several disadvantages, such as the difference in sensitivity and specificity among commercial diagnostic kits, the objectivity of the interpretation of the results, potential antibody cross-reactions, etc.

In the present study we searched several proteins from C. *burnetii* for their ability to differential diagnose acute or chronic Q fever in human blood serum. As a first step, 16 different proteins were chosen, according to literature for their antigenic properties. In addition, three proteins from Coxiella's Type IV secretion system (IcmG, IcmK and DotC) were chosen in order to be tested, since this nanomachine is important for the infection and pathogensis of *Coxiella*. By employing DNA cloning techniques, construction of different plasmids took place. Afterwards, proteins expression was tested in different conditions (culture temperature, IPTG concentration, time of culture after induction). When the proteins of interest were best

expressed, they were isolated and purified. The proteins were then used for the screening of sera from patients suffering from chronic Q fever endocarditis, patients whose samples were negative for phase I IgG, patients whose at least one sample was positive for phase I IgG and patients suffering from any kind of rheumatoid disease. Blood donors were used as the control group. Statistics were used to calculate sensitivity, specificity, positive predictive value, negative predictive value and Cohen's kappa coefficient (κ) and logistic regression binary analysis was also performed in order to find any possible combinations of proteins that could act as better diagnostic factors.

Keywords: *Coxiella burnetii*, Q fever, antigenic proteins, diagnosis, immunochromatographic kit.

Table of Contents

Abbreviations

bp: Base Pairs; **BSA:** Bovine Serum Albumin**; DDM:** n-Dodecyl β-D-maltoside**; Dot:** Defect in Organelle Trafficking; **Icm:** Intracellular Multiplication**; IPTG**: Isopropyl β-D-1-thiogalactopyranoside; **LDAO:** Dodecyldimethylaminoxid**; LAMP:** Lysosome-Associated Membrane Protein**; LC3:** Microtubule- associated protein light chain 3**; LCV:** Large Cell Variant**; LPS:** Lipopolysaccharide**; PAGE:** Polyacrylamide Gel Electrophoresis**; PCR:** Polymerase Chain Reaction**; PMSF:** Phenylmethylsulfonyl fluoride**; RAB:** Ras-Associated Binding; **SDS**:Sodium Dodecacyl Sulfate**;**
SCV: Small Cell Variant**;T4BSS:** Type 4B Secretion System**.**

1. INTRODUCTION

1.1 Prologue

Coxiella *burnetii* is an intracellular, Gram negative bacterium, that causes Q fever disease, a zoonosis which has a worldwide distribution with the exception of New Zealand and Antarctica [Lang, 1990, Herremants et al, 2013, Fournier, 1998] (Figure 1). During the past 25 years, 32 outbreaks in Europe have been identified, indicating that the number of Q fever cases is constantly increasing. Q fever has become a serious public health problem in many areas not previously known as endemic zones and represent a major health risk for humans and animals [Moseley et al, 2014].

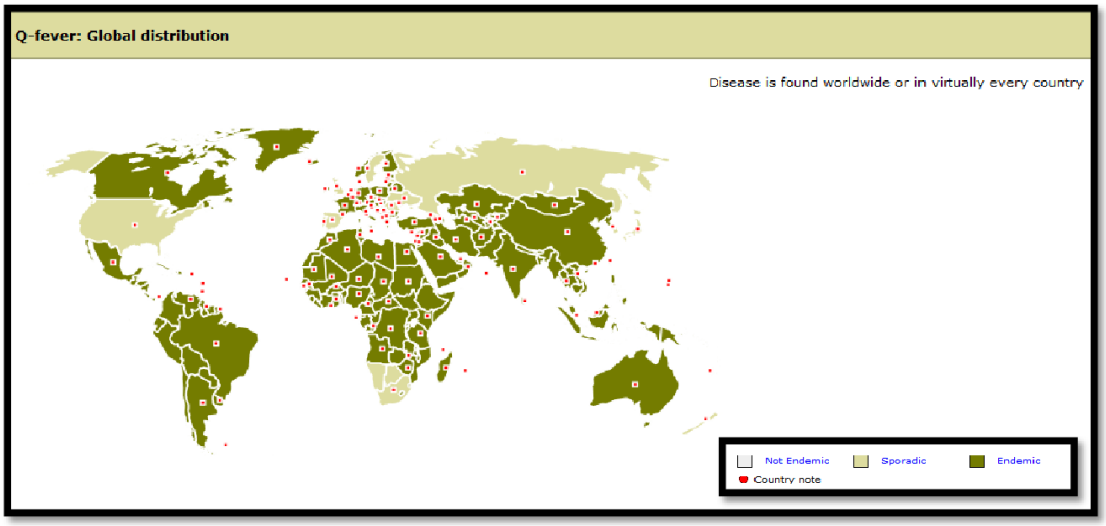

Figure 1: Global distribution of Q fever in 2014 [Tozer, 2015].

The disease in humans is thought to be contracted by direct contact with domestic animals like cattle, sheep or goats. Consequently, it is a disease largely associated with animal handlers. C.*burnetii* can survive for long periods of time in the soil and dust as a result of the dissemination from infected animals. Natural forces such as wind and dust movement, along with animal transportation have been considered to be secondary sources through which humans can be exposed to the bacterium and finally suffer from Q fever [Rodolakis et al, 2005, Parker et al, 2006].

1.2 History

Q fever stands for “Query fever” as very little was known about the causative agent and the disease when it was first discovered in the 1930s. Initially it was described in abattoir workers in Brisbane, Queensland, Australia by Edward Holbrook Derrick

[Raoult et al, 1999, Porter et al, 2011]. At the same time, the pathogen was isolated by Frank Macfarlane Burnet and Mavis Freeman from samples sent by Derrick, who hypothesized Q fever was of rickettsial origin [Roest et al, 2011].

Simultaneously, Herald Cox and Gordon Davis at the Rocky Mountain Laboratory in Hamilton, independently isolated a new infectious agent from ticks collected at Nine Mile Creek, Montana with rickettsia-like properties [Eldin et al, 2017]. A laboratory worker who became accidently infected by this new organism displayed symptoms remarkably similar to those of the Q fever that affected abattoir workers in Australia, suggesting a common infectious agent. Further studies in mice, confirmed these two newly discovered infectious agents to be the same pathogen [Eldin et al, 2017]. The pathogen was initially designated as *Rickettsia burnetii*, on account of its *Rickettsia*-like properties [Eldin et al, 2017]. Based on phenotypic characters, the pathogen was later classified under a new genus comprising of only one species, "*Coxiella burnetii*" in honor of the contribution made by Cox and Burnet.

1.3 Bacreriology of *Coxiella burnetii*

Coxiella burnetii is an obligate intracellular, small in size bacterium (0.2 to 0.4 μm wide, 0.4 to 1 μm long), the only species belonging to the *Coxiella* genus. In addition, the bacterium is classified in the γ-subdivision of proteobacteria, according to the sequencing of the 16s rRNA gene [Kazar, 2005]. *Coxiella*'s closest relatives appear to be *Legionella pneumonophila* and ricketsia. However, molecular techniques, and in particular genome sequencing, have released that Coxiella has a much closer genetic relationship to Legionella pneumophila than the *Rickettsia* [Kazar, 2005]. The existence of lipopolysaccharide (LPS) in its membrane, characterizes *Coxiella* as a Gram-negative bacterium. Although it can't be stained by Gram stain, it is stained by Gimenez stain (Figure 2A) [Abnave et al, 2017].

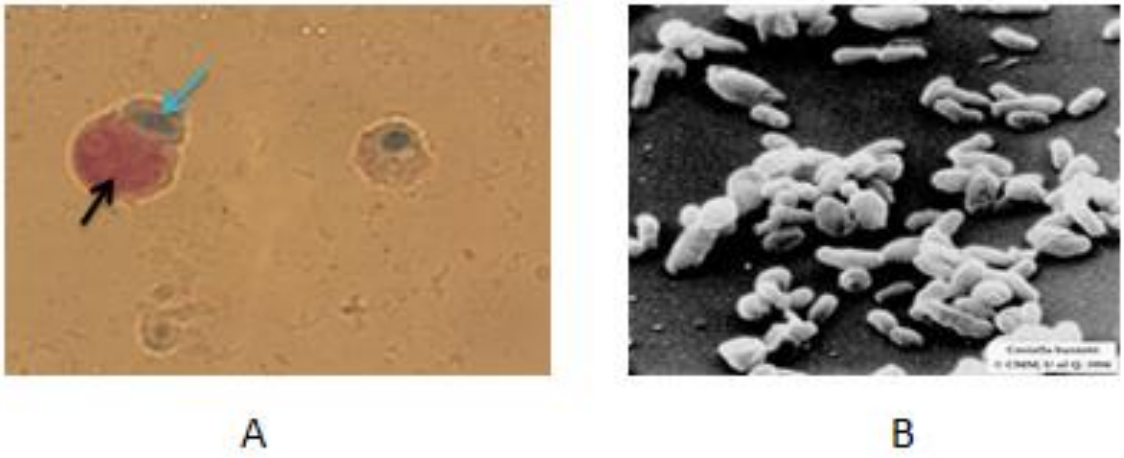

Figure 2: A. Gimenez stain in Vero cells infected by *C.burnetii*. *Coxiella's* cells are stained in pink colour (black arrow) while host cell is blue (blue arrow). The mature phagolysosome occupies 2/3 of the whole host cell. B.Electron microscopic image of *C.burnetii*, which causes the worldwide disease, Q fever [Samoilis, 2008].

1.3.1 Phase variation

Phase variation occurs among many species of pathogenic bacteria and allows the organism to avoid the host cells immune system.

A phase I lipopolysaccharide (LPS) structure of the bacterium is responsible for natural infection. It is a virulent form of *Coxiella burnetii* and it can infect animals, humans and arthropods. It is also characterized by full- length LPS and it is able to survive inside monocytes and macrophages [Raoult et al, 2002]. The phase I stage of the organism slowly changes after several reproductive passages to produce phase II. This form contains a chromosomal alteration which has genetic deletions in the gene responsible for LPS biosynthesis [Maurin, Raoult, 1999]. Therefore, phase II LPS displays an irreversible modification in its molecular weight. Phase II is avirulent and does not exist in the natural environment [Abnave et al, 2017].

According to Toman et al, the difference between the virulent and avirulent forms of *C. burnetii* lies in the existence of usuable sugars [Tomen et al, 1998]. In more details, LPS from virulent *C.burnetii* contains sugars, L-virenose, dihydrohydroxystreptose, and galactosamine uronyl-α-(1,6)-glucosamine residues, whereas LPS from the avirulent form does not have any of them. Both sugars (L-virenose and dihydrohydroxystreptose) have not been found in any LPS of other

enterobacteria, which means that they comprise the only biomarker for Coxiella *burnetii* [Narasaki, 2012].

1.3.2 Morphological Change

Coxiella burnetii can be found in two different cell variants; the small-cell-variant (SCV) and the large-cell-variant (LCV), when both of them are infectious. Morphologically, these two types of *Coxiella burnetii* cells have a lot of differences. According to many studies, the LCV has greater metabolic activity and is more sensitive to environmental stresses than the SCV, while the environmentally stable SCV has a thicker peptidoglycan layer, has more condensed nuclear material and, as the name implies, is smaller in size [Burtion, 1975, McCaul, 1981, McCaul, 1991].

In 2007, Coleman et al showed using Electron Microscopy that SCVs are rod shaped and 0.2 to 0.5 μm in length and have an electron-dense condensed chromatin with an array of intracytoplasmic membranes. On the other hand, LCVs can exceed 1 μm in length and are pleomorphic with a dispersed chromatin (Figure 3).

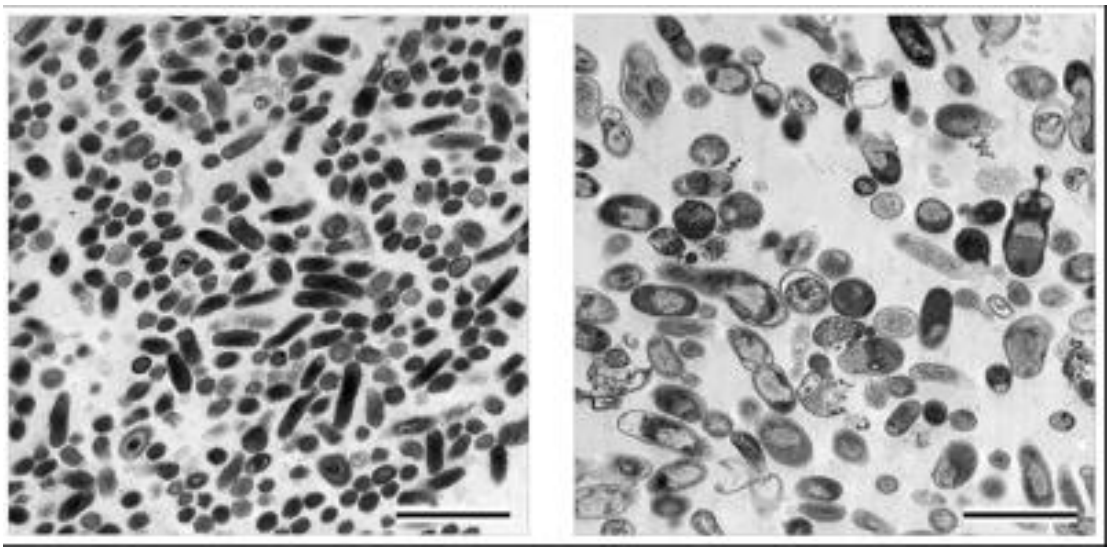

Figure 3: Transmission electron micrographs of SCVs (left panel) and LCVs (right panel) showing the characteristic size and structure of developmental forms. Bars, 2.0 μm. [Coleman et al, 2007].

Coxiella burnetii follows the usual bacterial growth cycle, including the lag, exponential and stationary phase. Based on electron microscopy studies each phase of the growth cycle signifies morphological differentiations for *Coxiella*. More specifically, Lag phase occurrs during the first 2 days postinfection (p.i.) and is primarily composed of SCV-to-LCV morphogenesis. LCV forms predominate over the

next 4 days, during which exponential growth is observed. Stationary phase begins at approximately 6 days p.i. and coincided with the reappearance of SCV, which increased in number at 8 days p.i. [Coleman et al, 2004, 2007]. This information indicates that the replicative form of the organism is the LCV, which multiplies inside the phagolysosome during the exponential phase of the growth. On the other hand, SCVs are metabolically inactive and highly resistant to environmental stress, such as osmotic pressure, heat and chemicals. Due to these features, SCVs can survive in the environment for long periods of time.

Expression of specific genes of *C. burnetii* is shown to be related with the growth phase of the host cell cycle, while there are proteins specific for both cell variants. For instance, ScvA and HQ1 are SCV-specific, DNA- binding proteins which play roles in chromatin condensation. These proteins are degraded during the lag and exponential phases, while they increase as Coxiella enters the stationary phase [Coleman et al, 2004]. On the other hand, elongation factors EF-Tu and EF-Ts, the stationary-phase sigma factor RpoS, and a protein with porin activity termed P1 are preferentially expressed by LCV [Coleman et al, 2004, 2007]. P1 is being upregulated during the exponential phase, a fact that agrees with the metabolic role of LCVs. This upregulation is presumably an adaptation by LCV to acquire nutrients from the lysosomal milieu.

Concluding all the above, it becomes obvious that each cell variant displays a different role during the infection. However, the molecular paths that control *Coxiella*'s cellular development are still undefined [Coleman et al, 2004, Coleman et al, 2007, Heinzen et al, 1999].

1.4 Life inside the host cell

C. burnetii is an intracellular pathogen that targets monocytes and alveolar macrophages. However, it also can infect different types of cells, such as epithelial cells and fibroblasts [Heinzen et al, 1999].

Once inside the phagocyte, *Coxiella* are contained within a phagosome. In the normal cellular destruction of foreign agents the phagosome matures, becoming acidic and fuse with secondary lysosomes to form phagolysosomes. This is the host cell's natural defense mechanism by which most foreign agents are destroyed

[Elhelu, 1983]. However, this is not the case with *Coxiella burnetii*. *Coxiella* bacterium causes the phagosome to form a large compartment known as a parasitophorous vacuole, which acts as the perfect acidic environment in which *Coxiella* can replicate [Voth and Heinzen, 2007]. This acidic, bacteria filled, vacuole remains non-toxic to the host cell [Klaassen et al, 2009].

The intracellular trafficking pathway of *Coxiella burnetii* inside the macrophage can be shown in Figure 4. More specifically, the bacterium binds to macrophages through $\alpha_v\beta_3$ integrin. This procedure triggers the phagocytosis of the bacterium through an actin-dependent mechanism. Five minutes after internalization, the nascent *Coxiella*-containing vacuole (CCV) acquires RAB5 and EEA1 and acidifies to approximately pH=5.4, which is characteristic of normal phagosomal development. By contrast to phagosomes, the CCV also acquires microtubule-associated protein light-chain 3 (LC3: an autophagosomal marker), a process that is dependent on bacterial protein synthesis. The nascent CCV develops through fusion and fission events with early endosomes and then late endosomes, leading to the disappearance of RAB5 and EEA1 and the acquisition of RAB7 and lysosome-associated membrane glycoprotein 1 (LAMP1). Forty to sixty minutes after internalization further acidification to pH=5 takes place, which is characteristic of normal phagosomal development. Lysosomal enzymes, including cathepsin D (CTSD), start accumulating in the CCV within two hours after internalization, at which point the vacuole is at pH ~4.5. This is delayed significantly from normal phagolysosomal acquisition of CTSD. This pause in CCV development might allow conversion of the bacteria from SCVs to the metabolically active LCVs. Between eight hours and two days after internalization, the CCV expands to occupy the whole cytoplasmic space of the host cell [Weber et al, 2013].

This process is dependent on bacterial protein synthesis and involves the recruitment of both RHO GTPase and RAB1B to the CCV membrane. RHO GTPase is likely to be involved in maintenance of the large vacuole, whereas the recruitment of RAB1B from the ER might facilitate the acquisition of additional membranes from host to create this spacious CCV.

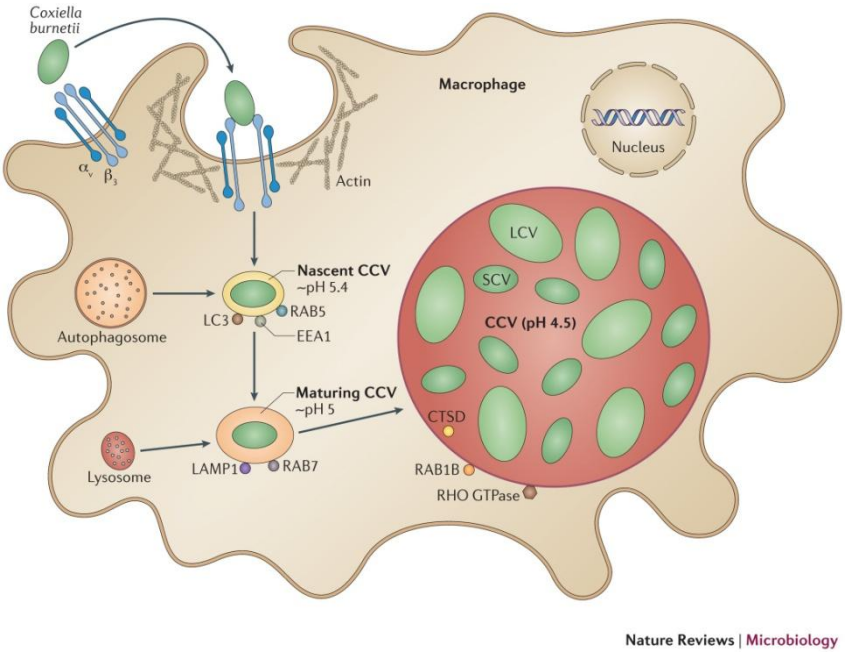

Figure 4: Biogenesis of *Coxiella*-containing vacuole (CCV). The pathogen occurs as a "spore-like" small cell variant (SCV) in the environment. Shortly after entering into a host cell, SCV transforms into the metabolically active large cell variant (LCV) within a lysosome-derived, acidic (pH ~4.5) *Coxiella*-containing vacuole (CCV). [Weber et al, 2013].

As many intracellular pathogens, *Coxiella burnetii* uses its proteins in order to invade and survive within host cells. In more detail, the ability of the pathogen to settle and replicate inside the host cell is dependent on the secretion of effector proteins that alter fundamental host cellular functions, which are associated with specific stages of infection [Voth and Heinzen, 2007, Moffat et al, 2015].

In general, protein secretion systems are found in almost all bacteria and they are used by pathogens to manipulate the host cell in order to get established within it [Harry H. et al, 2014]. Studies have shown that, eight different secretion systems have been identified [Depluverez et al, 2016]. Among them, Type IV secretion system (T4SS) is being used by some bacterial pathogens in order to deliver bacterial proteins directly to the cytoplasm of the host cell [Moffat et al, 2015]. *Legionella pneumonophila*, the closest relative of Coxiella *burnetii*, uses similar to Coxiella the T4SS in order to survive within the host cell.

Studies of Moffat et al in 2015 have shown that, in the case of *Coxiella burnetii* T4SS plays a crucial role in its survival and replication inside the macrophage. Several hours after *Coxiella* enters the host cell, effector proteins are being secreted to the

infected cell. When the expression of *Coxiella*'s genes, responsible for the protein markers Rab5 and Rab7 stops, secretion of effector proteins stops as well. Hence, we can conclude that the intracellular replication of the bacterium depends on T4SS.

1.5 Secretion System Type IV

Type IV secretion systems (T4SS) are nanomachines that are present in many species of gram-negative and gram-positive bacteria. T4SS form a channel between the bacterial inner and outer membranes in order to ordinate the delivery of molecules like proteins and DNA into bacterial and eukaryotic cell targets. In addition, T4SS are not only capable of secreting toxins which are lethal to other bacteria and mammalian cells, but also they act like drivers for antibiotic resistance spreading during bacterial conjugation. This is why T4SS can be exploited as drug targets against antibiotic resistance spreading and bacterial infectious diseases [Wallden et al, 2010].

VirB/VirD4 T4SS of the bacterium *Agrobacterium tumafaciens* is the archetype for T4SS and it is composed of 12 proteins. A. *tumafaciens* infects cell plants, causing a tumor- like disease, called crown gall [Yang Grace Li, Peter J. Christie, 2018]. This bacterium employs T4SS in order to deliver oncogenic DNA (Ti plasmid) and effector proteins to plant cells. *Coxiella burnetii* and *Legionella pneumophila* are microorganisms that employ T4SS. They are two bacteria, with similarities in their Type IV secretion system, with Coxiella's T4SS being very similar to that of *L. pneumonophila* [Kubori et al, 2014]. In fact, these two pathogens are the only bacteria known to date to use the IVB secretion system, also known as Icm/ Dot [Segal, 2005], a name that came from the initials of the words "Intracellular Multiplication" and "Defect in Organelle Trafficking" [Hoffman et al, 2008].

Coxiella encodes 24 out of 27 homologous proteins of Legionella's Dot/Icm secretion system [Molly C. et al, 2013]. The genes that encode those proteins are found on a 35Kbp region of *Coxiella* chromosome and they are arranged similarly to the Dot/Icm genes on *Legionella* chromosome [Seshadri et al, 2003].

Interestingly, according to the study of McGrath et al in 2003, many Dot/ Icm proteins are expressed during host cell infection. In fact, their expression precedes the formation of the phagolysosome. Consequently, T4SS is essential for the formation

of the parasitic vaculose of *Coxiella* and hence for the survival of the bacterium inside the host cell. In addition, other research [Hoffman et al, 2008] notes not only the excellent use of the secretion system in *Coxiella*'s intracellular replication but also the inhibition of host-cell death is necessary for the survive of *Coxiella* in the host cell.

The Icm/Dot secretion system proteins are organized into two subcomplexes, each of them playing a different role [Vincent et al, 2008]:

- Type IV coupling protein subcomplex: The Type IV protein coupling protein subcomponent is a complex of the inner membrane and consists of DotL (IcmO), DotM (IcmP), DotN (IcmJ) and the heterodimer pair IcmS/IcmW.
- Core transmembrane subcomplex: On the other hand, the core transmembrane subcomplex, is composed of the DotC, DotD, DotF (IcmG), DotG (IcmE) and DotH (IcmK) proteins. This subcomplex bridges both the inner and the outer membrane of the pathogens [Vincent et al, 2008]. In 2014, Kubori et al showed that this core transmembrane subcomplex has a ring shaped structure with a 40 nm diameter in *L*. *pneumolophila*. It has been demonstrated that DotC, DotD, and DotH (IcmK) are essential for the formation of the Dot/Icm core complex and form an ring-shaped outer-membrane subcomplex [Kubori et al, 2014]. In the absence of other components of the secretion system DotH (IcmK) remains unassociated with outer membrane. The outer membrane targeting of DotH (IcmK) depends on lipoproteins DotC and DotD, in a manner analogous with the function of pilotin in targeting secretin in type II secretion system [Vincent et al, 2006].

In Figure 5, Icm/ Dot secretion system of *Legionella pneumonophila* is being illustrated.

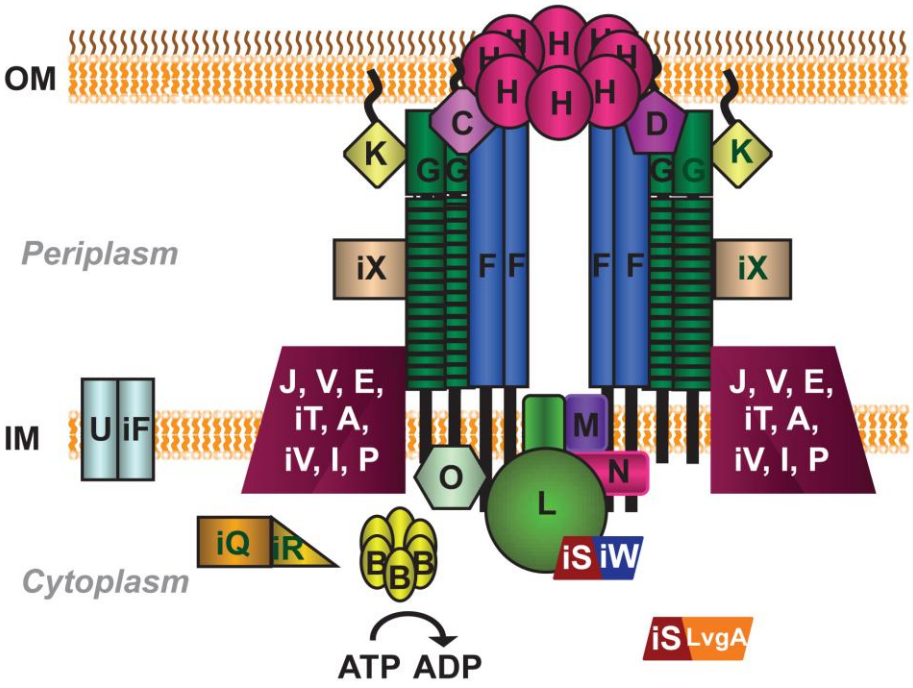

Figure.5: *L. pneumonophila* Icm/ Dot secretion system. Outer and inner membrane are showed, as well as the membrane and periplasmic proteins [Sutherland et al, 2013].

1.6 Q fever

Q fever is a worldwide zoonosis that is caused by the intracellular bacterium *C. burnetii.* Due to its surprisingly high infectious nature, *Coxiella* has been characterized as a world biological weapon agent, according to the Centers of Disease Control and Preventions, USA [Kagawa et al, 2003].

Domestic ruminants such as goats, sheeps and cattle are the primary reservoirs of the pathogen and represent the most frequent source of human infection [Woldehiwet, 2004]. Infections in animals are assumed to occur mostly by inhalation of the pathogen from the environment and are often asymptomatic [Roest et al, 2012]. The disease symptoms are usually manifested in pregnant animals and widely differ among hosts [Roeat et al, 2012]. For instance, in pregnant goats and sheep, the main clinical manifestations are abortions at final gestation stage. On the other hand, infected cattle usually do not show symptoms although abortions, subfertility and metritis are present in some cases [Arricau-Bouvery N, Rodolakis A., 2005]. Abortions in infected animals result in excretion of large numbers of *C. burnetii*

(10^9 CFU/g placenta) into the environment. Inhalation of contaminated aerosols from the environment are the main risk factor for infection in humans [Arricau-Bouvery and Rodolakis, 2005, Arricau-Bouvery et al, 2003]. Therefore, persons at highest risk for Q fever include farmers, veterinarians, abattoir workers, those in contact with dairy products, and laboratory personnel working with *C*. *burnetii*. Following infection, 60% of infected humans are usually asymptomatic.

1.6.1 Pathogenesis of the disease

As it was mentioned before, after exposure to the bacterium and infection, most of the cases (60%) remain asymptomatic. While *Coxiella* is being multiplied inside the host cell, immune response is taking place. Two or three weeks after infection, phase II antibodies can be detected [Teunis et al, 2013]. At the same time, the pathogen's DNA can be identified by PCR in patients serum, in the initial stages of the disease.

In humans, there are two different types of Q fever disease; the acute and the chronic type. At this point, it shoud be mentioned that the terminology "chronic Q fever" has been replaced by "persistent focalized *C.burnetii* infection", recently. Acute Q fever is mostly a self-limiting, mild, influenza-like disease, sometimes complicated by severe pneumonia or hepatitis. Among patients infected by C. *burnetii,* a proportion will progress to persistent focalized *C. burnetii* infection after primary infection [Porter et al, 2011]. This type of infection leads to high death rates if untreated, which makes early case finding and preventive measures critical for patients at high risk. Host factors, such as age, gender and certain medical conditions, may influence the disease presentations [Tissot-Dupont, H., 2007]. Age has been identified as a risk factor for persistent focalized *C. burnetii* infection, with a higher risk associated with increasing age [Eldin et al, 2017]. This was demonstrated in a Q fever outbreak in Switzerland in 1983, showing that humans older than 15 years had a five times greater risk of contracting the disease [Dupuis, 1986]. Similarly, a study in Greece showed an increase of confirmed clinical cases with an increase in age [Vranakis et al, 2012]. Pathogenesis of Q fever is being illustrated in Figure 6.

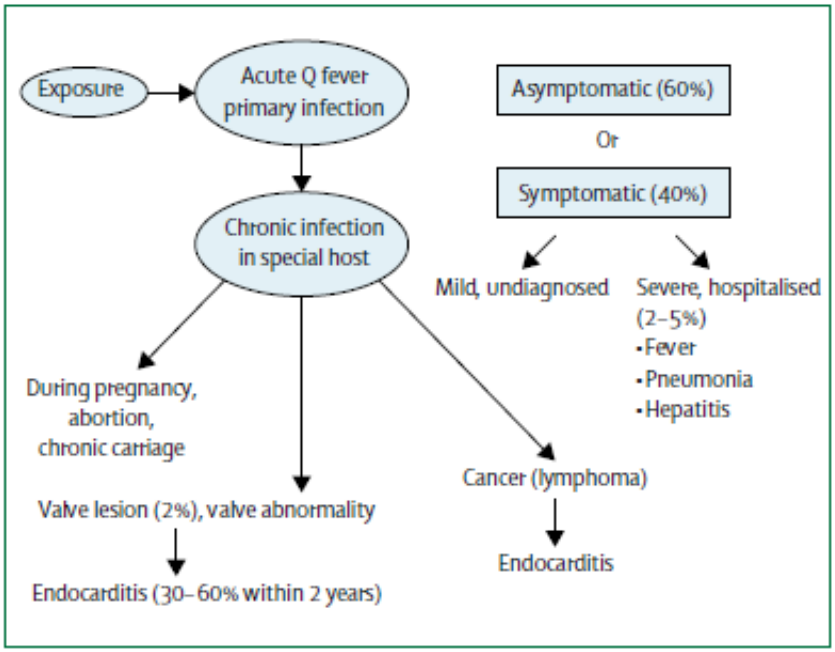

Figure 6: Pathogenesis of Q fever disease [Raoult et al, 2005].

1.6.2 Clinical disease states of Q fever

C. *burnetii* infections are asymptomatic or self-limiting, in their large majority. It is only when extensive laboratory investigations into Q fever are performed that it becomes evident that these patients have been exposed. Of those patients that acquire acute Q fever, 10-30% go on to develop chronic Q fever, in which 2% are diagnosed with endocarditis. The diverse range of clinical manifestations of the disease increases the misdiagnosed cases which in turn increases the mortality and morbidity associated with the disease [Nett et al, 2012].

- Acute Q fever

Acute Q fever infection is often asymptomatic, subclinical or cause extremely mild disease. It develops between two to five weeks of incubation period and it manifests symptoms to about 40% of the infected population. Acute Q fever disease symptoms range from high fever, myalgia, sweats and headaches to hepatitis, pneumonia, cardiac involvement andneurological involvement [Eldin et al, 2017].

- *C. burnetii* persistent focalized infections

5% of acute Q fever cases go on to develop chronic Q fever [Fenollar et al, 2001]. In these chronic infections, *C. burnetii* multiplies in host macrophages, producing a

permanent rickettsaemia, characterised by high levels of persistent antibodies. The heart is the most commonly affected organ, followed by the liver. Clinically, chronic Q fever presents as endocarditis in 60-70% of cases, but the arteries, bones and liver may also be affected [Maurin and Raoult, 1999]. Endocarditis usually occurs in patients with male sex, age above 40 years, and, most importantly, underlying valvular heart disease, even if clinically silent at diagnosis. Having a valvular prosthesis (mechanical or bioprosthesis) represents the most important risk factor [Eldin et al, 2017]. Chronic Q fever may also be present with vascular infections, osteorticular infections and persistent focalized lymphadenitis [Eldin et al, 2017].

1.7 Diagnosis of Q fever

1.7.1 Serological Tests

The diagnosis of Q fever is based on the detection of specific antibodies produced by hosts against C. *burnetii* after exposure. Until relatively recently, the complement fixation test (CFT) and the micro agglutination assay were the methods used in the detection of antibodies against *C. burnetii* [Roest et al, 2013]. However, the indirect immunofluorescence assay (IFA) is now considered the reference method or “Gold standard” for the diagnosis of Q fever by serology [Harremans et al, 2013].

In this method, patient serum samples are tested for the detection of two biomarkers: the acute antibody marker immunoglobulin M (IgM) and immunoglobulin G (IgG) [Hunt J. et al, 1983]. In IFA an IgG anti-phase II antibody titer of ≥200 and an IgM anti-phase II antibody titer of ≥50 are generally considered significant for the laboratory diagnosis of acute Q fever [Eldin et al, 2017]. 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. However, the choice of cut-off titers depends on the amount of background antigen stimulation in the population under study and varies from one area to another [Maurin and Raoult 1999], [Angelakis and Raoult 2010). In addition, this IFA method appears to be subjective and there have been studies performed to measure inter-laboratory variation, with one showing only a 35% agreement rate between laboratories [Healy B. et al, 2011]. Moreover, this technique has several disadvantages, such as the requirement of acute and convalescent sera, the

objectivity of the interpretation of the results, potential antibody cross-reactions (false positive samples), the need of experienced personnel, etc [Vranakis et al, 2019].

Meanwhile, there are some other systems designed to measure antibody levels which also have high sensitivity and specificity such as the enzyme linked immunosorbant assay (ELISA). The advantage of ELISA is that it is easy to perform, interpretation is less subjective than for IFA and CFT, and automation is possible [Eldin et al, 2017]. However, commercially available Q fever ELISA tests lack the ability of IFA to identify patients at risk for developing chronic Q fever. In addition, they show large differences in sensitivity and specificity and they are not limited in the distinction between acute, chronic and past disease [Herremans et al, 2013].

1.7.2 Molecular Techniques

Molecular methods are used in the detection of many bacterial and viral pathogens and have become the gold standard of testing for many infectious agents [Schuller et al, 2010]. The development of molecular methods such as the polymerase chain reaction (PCR) and the continuing advances made by molecular diagnostics such as sequencing whole genomes, have improved the early and accurate diagnosis of acute Q fever, along with the ability to confirm chronic cases of Q fever.

There have been many PCR assays designed for the detection of *Coxiella*'s DNA, and these have targeted different areas of the organism's genome [Turra et al, 2006]. The target most often chosen to detect *Coxiella*'s DNA is the repetitive insertion element sequence IS1111, which has multiple copies throughout *Coxiella*'s genome [Klee et al, 2006]. The Com1 gene is the another commonly selected gene target which encodes for a 27-kDa outer membrane protein [Howe et al, 2009] . PCR has also allowed the detection of Q fever disease from a variety of different sample types, including serum. Acute Q fever is often diagnosed by PCR in the very early stages of disease using sera that has tested negative to *Coxiella* antibodies [Turra et al, 2006]. PCR has also been used widely for the detection of *Coxiella*'s DNA in chronic Q fever patient samples. These generally are tissue samples taken directly from infected organs which highly loaded with bacteria; for example: heart valves in the cases of endocarditis [Hartzell et al, 2008].

The diagnosis of acute Q fever is as crucial as the differential diagnosis between the acute and the chronic type of the disease, because the transition from acute to chronic Q fever could involve complications of the illness (usually endocarditis) as well as the need of antibiotic treatment.

1.8 Treatment and prevention

1.8.1 Treatment of Q fever

Infection by *Coxiella burnetii* is often unrecognized or misdiagnosed because of the wide range of its symptoms that are similar to those of many other common illnesses such as the flu [Richardus et al, 1987]. Most acute infections are subclinical and recover without medical intervention. Doxycycline is the antibiotic of choice because of its ability to permeate cell membranes and destroy the intracellular organism within a few weeks [Watanabe and Takahashi, 2008].

Chronic Q fever disease is much poorer in prognosis [Raoult et al, 1990]. This illness persists for longer periods of time and has a mortality rate of up to 60% [Raoult et al, 1999]. Endocarditis is the main clinical manifestation of chronic Q fever and is much more difficult to treat effectively, requiring a combination of antibiotics, usually: Doxycycline in combination with hydrochloroquine for 1.5 to 3 years.

Successful treatment is characterized by the decreasing of IgG and IgA phase I antibodies to < 200. In addition, clinical and biological evaluation should be performed at least annually for the rest of the patient's life [Maurin and Raoult, 1999].

1.8.2 Vaccine

The primary method of preventing Q fever is through vaccination of humans and animals. During initial vaccine development, the antigenic nature of C. *burnetii* was unknown. Later, it was proven that the potency and the efficacy of the vaccine were dependent upon the antigenic phase of the organism [Ormsbee et al, 1964]. Vaccines developed with phase I organisms have a potency of 100-300 times that of cellular vaccines produced using the phase II organism [Ormsbee et al, 1964]. In Australia, a whole cell vaccine against Q fever "Q Vax" was manufactured by the Commonwealth Serum Laboratory (CSL), and was licensed for use in 1989. This vaccine was implemented for wide-spread use into a government funded vaccination program with the aim to substantially reduce the number of Q fever infections

associated with occupational outbreaks. Australia is the only country in the world that has a licensed vaccine for the prevention of Q fever in humans.

In regard to Europe, after the outbreak of Q fever in France, Germany and the Netherlands in 2009, restricting the disease in animals became a priority. There are currently two commercially available vaccines against C. burnetii in ruminants. The first one to be developed is an inactivated bivalent vaccine prepared from Chlamydophila Q Fever Vaccination in Ruminants: A Critical Review 371 abortus and phase-II of C. burnetii (Chlamyvax FQ®, Mérial, France). It is indicated for sheep and goats. Since 2004, a second vaccine has been available. It is an inactivated nonadjuvanted phase-I C. burnetii antigen Nine Mile strain vaccine (Coxevac®, CEVA Santé Animale, France) and this vaccine is indicated for goats and cattle. Vaccination of sheep with this vaccine is considered off-label use [Achard and Rodolakis, 2017]. According to the study of Achard and Rodolakis of 2017, vaccination of ruminants with a phase-I vaccine is a safe and efficient strategy to prevent or limit the expression of Q fever in a herd. In infected herds, the long term administration of the vaccine to a majority of animals seems to be the best course of action to eradicate Q fever. Additional medical and hygienic measures can also be taken to improve the control of the disease.

New generation of Q fever vaccines has been focused on whole deactivated cells with removing the factors responsible for sensitivity, such as LPS [Kazar et al. 1982, Williams et al. 1986, Fries et al. 1993, Waag et al. 1997].

1.9 Aim of this study

According to all mentioned above, *C. burnetii* is the causative agent of the worldwide zoonosis Q fever. This pathogen can also infect humans, inducing two different types of the disease; the acute and the chronic. The current “gold standard” method for Q fever diagnosis is IFA method. However this technique appears to have some important technical disadvantages, when it comes to diagnosis by usind commercial diagnostic kits. For this purpose, the development of a new and reliable diagnostic tool is really crucial.

Consequently, the major goal of this current study is the development of a reliable serological diagnostic tool for the differential diagnosis of Q fever, based on ELISA

technique. Several proteins were chosen, after literature investigation, as potential biomarkers. We chose to study antigenic proteins that have been described as specific for acute or chronic Q fever, respectively. In addition, three proteins from the T4SS of *Coxiella* (IcmG, IcmK and DotC) were chosen in order to be tested for their ability to differential diagnose Q fever.

After choosing the proteins of our interest, we proceeded to cloning techniques in order to construct plasmids including the respective genes. Then, the recombinant antigenic proteins were expressed, isolated, purified and tested with Western Blot and ELISA by using different serum samples from patients suffering from chronic Q fever endocarditis, patients whose samples were negative for phase I IgG, patients whose at least one sample was positive for phase I IgG and patients suffering from any kind of rheumatoid disease. Blood donors were used as the control group. Finally, all ELISA results were statistical analyzed and conclusions were made.

The outcome of this study could be really beneficial not only for the scientific community but also for the public health.

2. MATERIALS AND METHODS

List of the Chemicals used and their Companies

Albumin biotin-free: Roth

Albumin: Roth

Avidin: Cerbu biotechnik

BCA Reagent A: Pierce

BCA Reagent B: ThermoScientific

BCIP-NBT: ThermoScientific

Carbenicillin: Cerbu biotechnik

Coomassie Brilliant Blue G-250: Thermoscientific

DDM: Glycon Biochemicals

Desthiobiotin: IBA

EDTA: Roth

Ethanol: Fischer

Gene Ruler: ThermoScientific

Glycerol: Roth

HABA (2- [4'-hydroxy-benzeneazo] benzoic acid): Fluka 43

HEPES: Roth

IPTG: Cerbu biotechnik

K_2HPO_4: Fluka

KH_2PO_4: Fluka

KCl: Fluka

MOPS: Roth

NaCl: Analar Normapur

Ni-NTA column material: QIAGEN

PMSF: Cerbu biotechnik

Precast gels: Invitrogen

Prestained Marker: ThermoScientific

SDS: Roth

Streptactin column material: IBA

Tris: Roth

Tryptone: Roth

Tween: Roth

Yeast Extract: Fluka

2.1 Choosing *Coxiella burnetii's* genes of interest

According to the aim of the current study, we have tried to find potential biomarkers for the differential diagnosis of Q fever disease. For this purpose, literature was screened and some antigenic proteins of *Coxiella burnetii* were chosen as potential diagnostic tools of the chronic and the acute type of the disease, respectively. In addition, we chose 3 membrane proteins from *Coxiella*'s type IV secretion system, in order to study their ability to differential diagnose Q fever. The proteins which were tested are listed in Table 1.

Table 1: Proteins investigated in the current study for their ability to diagnose acute or chronic Q fever.

Number	Gene Name	Gene length (bp)	Protein name	Molecular Weight (KDa)	Specificity (according to literature)	Localization
1	Com1	771	CBU_1910	27,6	Chronic Q fever	Outer membrane
2	HspB (GroEl)	1,671	CBU_1718	60		Periplasm
3	YbgF	930	CBU_0092	34,2		Periplasm
4	UPF0422	1,410	CBU_0937	51,38		Outer Membrane
5	Tuf-2	1,206	CBU_0236	43,52		Cytoplasm
6	OmpH	510	CBU_0612	18,8		Outer Membrane
7	Ssb	490	CBU_0271	17,43	Acute Q	Unknown

8	-	312	CBU_0632	11,88	fever	Unknown
9	-	693	CBU_0952	25,95		Unknown
10	Omp1	768	CBU_0311	26,77		Outer Membrane
11	rplL	390	CBU_0229	13,24		Cytosole
12	-	948	CBU_0891	34,37		Membrane
13	yazC	360	CBU_1143	12,52		Inner Membrane
14	LemA	657	CBU_0545	24,75		Membrane
15	sucB	1.226	CBU_1398	45,88		Unknown
16	IcmG	749	CBU_1626	26,56	Unknown	Transmembrane
17	IcmK	1.070	CBU_1628	37,43		Outer Membrane
18	DotC	839	CBU_1644	30.27		Membrane

2.2. Construction of plasmids for the expression of the recombinant proteins

For the constructions of plasmids for the targets 1-15 (Table 1), the cloning strategy followed was identical. Genomic DNA from *Coxiella burnetii* was isolated using the Qiamp Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. Primers (Table S1) were designed based on the genome sequence of *C. burnetii* RSA 493/Nine Mile phase I (NCBI: NC_002971). Fourteen full-length genes (CBU_1910, CBU_1718, CBU_0092, CBU_0937, CBU_0236, CBU_0612, CBU_0271, CBU_0952, CBU_0311, CBU_0229, CBU_0891, CBU_1143, CBU_0545 and CBU_1398) were amplified by PCR using Phusion DNA polymerase (Thermo Fisher Scientific). The PCR amplified products were purified from the agarose gel using Zymoclean Gel DNA Recovery kit (Zymo Research) and then digested with NdeI and XhoI. Subsequently, the digested fragments were ligated into the corresponding sites of pET-22b(+) to generate the expression vectors. In addition, for the target CBU_0937, two primers (CBU_0937_pelB_Fw and CBU_0937_pelB_Rev) were used to amplify the region encoding the mature protein without the first 23

amino acids according to the predicted signal peptide cleavage site. The resulting DNA fragment was cloned into the NdeI and XhoI sites of the pET-22b(+) vector using the InFusion ligation-independent cloning method (Takara Bio). All final constructs were verified by DNA sequencing (Eurofins Genomics).

For the expression of the sufficient amount of the rest of the targets in a form with high purity, we tried tree different constructs, concluding that pTTQ18-C3 for DotC and pTTQ18-C-strep for IcmG and IcmK were the most efficient.

Construction of the plasmid pTTQ18-A2.

PCR was performed (Phusion NEB M0530) with gene specific primers, including enzyme recognition sites from *Coxiella*'s genomic DNA (*C. burnetii*, strain RSA 493/Nine Mile phase I, GenBank /NCBI). The primers were used at final concentration of 10pmol/μL. The PCR products were cloned into pJET1.2/blunt (CloneJET PCR Cloning Thermo #K1232). They were analyzed by restriction digest, using the restriction enzymes BamHI and EcoRI and cloned into the destination vector, pTTQ-18 by ligation. The plasmid was transformed into DH5α *E. coli* cells. Restriction digest was performed on the constructs with BamHI and EcoRI, in order to determine which clones contained the correct inserts. Plasmid DNA from those constructs was analyzed by DNA sequencing (Eurofins Genomics).

Construction of the plasmid pTTQ18- C3.

The plasmid was constructed by restriction digest of pTTQ18-A2 with BamHI and EcoRI. The fragments, including *C. burnetii*'s proper gene, were ligated into pTTQ18-C3 plasmid, which was also digested with the same enzymes. Transformation into DH5α *E. coli* cells produced clones, whose plasmids DNA were isolated and analyzed by DNA sequencing (Eurofins Genomics).

Construction of the pTTQ18-C-strep plasmid.

PCR and religation were performed with specific primers, listed in Table S1, in order to delete the N-terminal His and Tev of construct pTTQ18-C3. Either the PCR product or the PCR primers needed to be phosphorylated for religation. The new constucts were transformed into DH5α *E. coli* cells and plasmid DNA from the clones contained the inserts were analyzed by DNA sequencing (Eurofins Genomics).

These constructs were designed pTTQ18-C3-C, pTTQ18- G-C-strep and pTTQ18-K-C-strep, respectively.

Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) is a method which allows the amplification of a DNA region. For this purpose, two oligonocleotides or primers are designed to match DNA sequence at either end of the region of interest. Primers designed for our targets are listed in Table S1. The first stage of PCR is denaturation and it takes place at 94^{o}C for 3 minutes, in order to denaturate the DNA template and to make the single strands accessible for the primers to anneal. Second is the annealing step, carried out at around 56^{o}C (it depends on the primers) for 30 s, which allows the primers to bind to the section of template DNA they have been designed to complement. Third is the extension phase at 72^{o}C, which enables the Taq polymerase I to synthesise new strands of DNA complementary to the template, beginning at the points where primers have bound. The time of the extension phase depends on DNA sequence of interest length (15-30 s/Kb). This is followed by further cycles of the three temperature phases, resulting in an exponential increase in the number of copies of the amplified DNA fragment.

Agarose DNA gels

Agarose gels were prepared by adding 1,5% (w/v) Agarose in 1xTAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.2- 8.4). Samples were prepared by mixing with DNA running buffer (6x: 10 mM Tris-HCl (pH 7.6) 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol FF, 60% glycerol, 60 mM EDTA - ThermoFisher Scientific) to a final concentration of 1x. 5-10µL of DNA samples were loaded per well (small comb) or up to 50µL (large comb). Gels were run in a tank containing 1x TAE for about 45 minutes at 150 V. After running, gels were immersed in Ethidium Bromide solution (0.5% v/v in water) and then bands were visualized by UV lamb.

DNA digestion

Restriction digestion of plasmids for cloning was performed in order to check if the correct insert was cloned in the vector. The corresponding reaction is being described below:

- 0,5-1μg/μL DNA
- 1 μL buffer (suitable for the restriction enzyme) (10x)
- 1 μL restriction enzyme (10 U/μL)
- Nuclease- free water , to final volume equal to 10μL

Reactions were incubated at 37^{o}C for 30 minutes and then they were analyzed by agarose gel.

Ligation

Fragments to be ligated were double digested with the same restriction enzymes. Restriction digested fragments were ligated together in a 10 μL ligation reaction (NEB- Quick Ligation Kit) containing:

- 5 μL Quick Ligase Reaction Buffer (2x)
- X μL Vector (50 ng)
- Y μL insert (3x molar vector concentration)
- 1 μL Quick Ligase
- Nuclease- free water, to final volume equal to 10μL.

The reaction was incubated for 15 minutes at room temperature. The ligation reaction product was then transformed in DH5α competent cells.

2.3 Growth medium for *E. coli* cultures

To prepare 1 L of LB medium (10 g Tryptone, 5 g Yeast extract, 10 g NaCl), the proper amounts of the chemicals are dissolved in 900 mL of distilled water. The pH of the solution is being adjusted to 7,0-7,2 by adding sodium hydroxide (NaOH) solution (1 N). Once the pH is adjusted, distilled water is used to fill the solution to the final volume of 1 L. LB medium is then sterilized in autoclave (120^{o}C, 15 psi, 20 minutes).

For the preparation of LB agar plates (solid culture plates), 15 g of agar are dissolved in 1 L of LB medium and sterilized by autoclaving. After cooling to about 50^{o}C, the proper amount of carbenicillin is added to a final concentration of 50 μg/mL (1:1000

dilution of the stock solution). The solution is poured into Petri plates and stays at room temperature until it solidifies. LB agar plates are stored at 4^{o}C.

2.4 Transformation

Bacterial transformation is a key step in molecular cloning, the goal of which is to produce multiple copies of a recombinant DNA molecule. The two most popular methods of bacterial transformation are (1) heat shock of chemically prepared competent cells (chemical transformation), and (2) electroporation of electrocompetent cells. In this study, all the transformation experiments were performed by the heat shock method.

Fifty μL of *E*. *coli* competent cells are mixed with 0,5 μL (1 pg – 100 ng) of plasmid DNA and briefly exposed to an elevated temperature, a process known as heat shock. First, cells are incubated with DNA on ice for 10 minutes in an eppedorf tube. Heat shock is performed at 42°C for 40 seconds. Then, heat-shocked cells are returned to ice for 10 minutes. Following heat shock, transformed cells are cultured in 1 mL of prewarmed SOC medium (2%(w/v) Tryptone, 0.5%(w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM Glucose) at 37°C with shaking at 225 rpm for 1 hour. After regenerating in SOC medium, the cells are plated in LB agar plates containing 50 μg/mL carbenicillin. The plates are incubated overnight at 37^{o}C and they are examined the next day for colonies formation.

2.5 *E. coli* cells cultures

After bacterial transformation and successful colonies formation, one single colony is picked from the plate and is inoculated into 3 mL of LB medium with the proper amount of carbenicillin (final concentration of 50 μg/mL). This preculture is incubated at 37^{o}C with shaking at 225 rpm for 8 hours. Afterwards, it is inoculated into 1 L LB medium with the proper quantity of the antibiotic. This is the main culture and it is used for the expression of the recombinant proteins. When the optical density at 600 nm (OD_{600}) of the main culture reach 0.5-0.7, induction is performed (with IPTG) and then the cells continue to grow for 2-4 hours (Each recombinant protein expresses itself better in different culture time after induction). Then, cell pellet is being harvested with centrifugation of the culture at 1,0000x*g* for 15 minutes in an Avanti J-26XP, Beckman centrifuge and it can be stored at -20^{o}C.

2.6 Cell lysis

Cell pellet is being resuspended into resuspending buffer (20 mM HEPES, 300 mM NaCl,1 mM EDTA pH 8). The volume of the lysis buffer that is used equals to six times the total cell pellet mass. When the cells are resuspended, a few mg of DNAase and 1 mM of PMSF are added. Cells are being broken either by French Press (SLM Aminco, Model: FA-078) at 40,000 PSI or by Microfluodizer (Microfluidics Corporation) at 80,000 PSI.

Broken cells are centrifuged at 10,000x*g*, 4^{o}C for 20 minutes. The pellet is discarded and the supernatant is ultracentrifuged at 200,000x*g*, 4^{o}C for 90 minutes in a 70Ti Beckmann rotor (L-90K Beckman Coulter). Membrane pellet is being resuspended in the lysis buffer and its protein concentration is being measured by Brandford Assay (paragraph 2.7). Both the membrane fraction and the supernatant are stored in -80^{o}C for further use.

2.7 Brandford protein Assay

The Bradford protein assay is one of the several methods commonly used to determine the total protein concentration of a sample [Noble and Bailey, 2009]. The method is based on the proportional binding of the dye Coomassie Brilliant Blue to proteins. Within the linear range of the assay, the more protein is present, the more Coomassie binds. Furthermore, the assay is colourimetric; as the protein concentration increases, the colour of the test sample becomes darker. Coomassie absorbs at 595 nm, when bound to protein. The protein concentration of a test sample is determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in this assay. Although different protein standards can be used, Bovine Serum Albumin (BSA) is chosen as the standard.

The working solution is Brandford Reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) Ethanol, 8.5% (w/v) Phosphoric acid). The first step of Brandford assay is the creation of the standard curve. For this reason, standard concentrations of BSA of 1, 5, 7.5 and 10 μg/mL as well as a blank sample (only buffer and brandford solution) are being prepared. Standard curve is being used for the calculation of the final protein concentration in the samples. Afterwards, the samples

are being prepared and after 5 minutes the absorbance at 595 nm is being recorded. Protein concentration is then being calculates according to standard curve in mg/mL.

2.8 BCA protein Assay

The BCA Protein Assay is another popular method for colourimetric detection and quantitation of total protein. Furthermore, BCA Protein Assays have an advantage over the Coomassie dye–based assays (Bradford)–they are compatible with samples that contain up to 5% surfactants (detergents), and are affected much less by protein compositional differences, providing greater protein-to-protein uniformity.

Different volumes (1-10μL) of the BSA stock solution (1 mg/mL) are added in a 96-well plate, in order to create the standard curve. Ten μL of unknown samples in different dilutions (if needed) are added in the appropriate wells. Afterwards, BCA solution is made by mixing Reagent A (Bicinchoninic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1 N NaOH, final pH 11.25) with Reagent B (4%$CuSO_4$ x 5 H_2O) in ratio 1:50 respectively. 200 μL of BCA solution are added to the standard curve samples and the unknown ones. All measurements and protein concentration calculations are done by Tristar LB 941 microplate reader (Frankfurt am Main, Germany).

2.9 Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis is the separation of macromolecules in an electric field. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides. Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed.

Materials

- AB- mix buffer (49,5% T, 3%C)
  - Acrylamide 48 g / 100 mL
  - Bis-acrylamide 1,5 g/ 100 mL

- Gel Buffer (3x)
  - 3M Tris/HCl, pH 8.45
  - 0.3% w/v SDS

- Glycerol (100%)
- Ammonium Persulfate (APS) 10% w/v
- TEMED

Solutions

Sample Buffer (2x)

- SDS, 12% w/v
- Glycerol, 10% w/v
- Mercaptoethanol, 6%
- Coomasie Brilliant Blue G-250, 0,05%
- Tris/HCl, 100mM, pH= 7.15

Running Buffers

- Anode Buffer, 10x
  - 1 M Tris/ HCl, pH 8.9

- Cathode Buffer, 10x
  - 1 M Tris
  - 1 M Tricine
  - SDS, 1% w/v

    pH 8.25

Staining Solution

- Phosphoric acid, 10% v/v
- Ammonium Sulfate, 10% w/v
- Coomassie Brilliant Blue G-250, 0,12% w/v
- Methanol, 20%

Destaining solution

- Acetic acid, 7% v/v
- Methanol, 5%
- ddH_2O

Preparing SDS- PAGE gels

Stacking gel, 4% (Total Volume of 12 mL):

- 1 mL AB- mix
- 4 mL Gel Buffer, 3x
- H_2O (to final volume 12 mL)
- 90 μL 10% APS
- 9 μL TEMED

Separating gel, 12% (Total Volume of 30 mL):

- 6 mL AB-mix
- 10 mL Gel Buffer 3x
- 3 g Glycerol 100%
- H_2O (to final volume 30 mL)
- 150 mL APS
- 15 μL TEMED

Polyacrylamide gels are polymerized after adding APS and TEMED in proper amounts. Protein samples are treated with sample buffer and heated at 40^{o}C for 20 minutes. SDS-PAGE is taken place in low electricity voltage (50-60 V) until samples overcome stacking gel. Then, the voltage is increased up to 120 V. After the end of electrophoresis, gels are stained in dye solution and protein bands begin to appear after 3 hours of staining.

2.10 Immunoblot (Western Blot)

Western blot is used to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with antibodies specific to the protein of interest.

After SDS-PAGE, protein bands are transferred from polyacrylamide gel into a PVDF membrane. This procedure takes place in 7 minutes, using i-Blot device (Invitrogen, Frankfurt am Main, Germany). Afterwards, the membrane is being blocked for 1 h with 2% (w/v) albumin biotin in TBST (10 mM Tris/HCl, pH 8, 150 mM NaCl, 0.05% (w/v) Tween 20) Blots are then incubated for 1 h with anti-polyHistidine-Alkaline Phosphate antibody (Sigma) or anti-strep-Alkaline Phosphate antibody at a 1:2000 dilution in TBST. Finally, the blots are developed using BCIP/NBT system. BCIP molecule is hydrolyzed by AP Buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and the produced molecule dimerized to give a colourful product, where the protein of interest was. In addition, in this study we used sera from patient diagnosed with acute or chronic Q fever, in order to test our proteins of interest ability to correctly identify chronic or acute Q fever.

2.11 Blue Native PAGE

Blue native polyacrylamide gel electrophoresis is a very common method implicated for characterization of oligomeric state of proteins. In this method, a dye, Coomassie Brilliant Blue-G250, is used for induction of external negative charge on the protein complexes. The complexes are then separated based on their molecular weights. In BN-PAGE, the protein complexes tend to migrate according to the pore size of the gradient gel till they reach the pore size limit point.

After preparing the samples with the addition of sample buffer (50 mM Bis Tris, 6 N HCl, 50 mM NaCl, 10%(w/v) Glycerol, 0.001% Ponceau S, pH 7.2), they are loaded in gradient 4-16% Native PAGE Bis-Tris Protein Gels (Invitrogen). The gels run at 4^oC in 150 V for 1 h and then in 250 V for 30 min. Anode buffer consists of 2.5 mM Bis Tris and 2.5 mM Tricine, pH 6.8 (1x running buffer), while cathode buffer consists of 1x running buffer and 0.02% (w/v) Coomasie G-250. Afterwards, the gels are soaked in distaining buffer (8% CH_3COOH), overnight at room temperature.

Standard proteins for Blue Native Page marker:

IgM Hexamer	1,236KDa
IgM Pentamer	1,048KDa
Apoferritin band 1	720KDa
Apoferritin band 2	480KDa
B-phycoerythrin	242KDa
Lactase Dehydrogenase	146KDa
Bovine serum albumin	66KDa
Soybean trypsin inhibitor	20KDa

2.12 Membranes solubilization by using n-dodecyl-β, D maltoside (DDM)

The membrane fraction is solubilized by DDM, in a final concentration of 1% w/v in buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8 and 1 mM PMSF. The final protein concentration in this solution should be 5 mg/mL. Membranes are being solubilized at 4^{o}C for 1 hour with slow stirring. In case recombinant proteins carry a Strep-tag, avidin is used for membrane solubilization in final concentration of 0,1 mg/mL. Solubilized membranes are centrifuged in 200,000x*g* for 1.5 hours, 4^{o}C in a L-90K Beckman Coulter Ultracentrifuge. Supernatant in being collected for further study.

2.13 Protein purification- Affinity chromatography

Affinity chromatography is a separation process used to purify molecules or a group of molecules that are in a biochemical mixture. It employs two phases: a stationary phase and a mobile phase. Specific molecules from the mobile phase will bond to the stationary phase based on their properties, whilst the rest of the solution is passing through unaffected.

Affinity purification involves 3 main steps:

A) Incubation of a sample with the affinity support to allow the target molecule in the sample to bind to the immobilized ligand.

B) Washing away non-bound sample components from the support.

C) Elution (dissociation and recovery) of the target molecule from the immobilized ligand by altering the buffer conditions so that the binding interaction no longer occurs.

2.13.1 Ni^{+2} – NTA column

The His-tagged recombinant proteins are purified by Ni-NTA affinity chromatography. Soluble fractions and solubilized membranes are loaded onto a 4 ml Nickel-NTA column, which was pre-equilibrated with 10 column volums of equilibration buffer (20mM HEPES, pH= 7.5, 300mM NaCl, 10mM imidazole). After loading the sample, two washing steps (10 column volumes) of 10 mM and 30 mM of imidazole respectively are performed. The target proteins are eluted with 5 column volumes of elution buffer (20 mM HEPES, pH 7.5, 300 mM NaCl, 300 mM imidazole).

2.13.2 Streptactin column

Streptactin affinity chromatography is used to purify Strep-tagged recombinant proteins. Soluble fractions and solubilized membranes are filtered through a 0.45 µm syringe filter (Rephile) and loaded onto the streptractin affinity column, which has been equilibrated with equilibration buffer (150 mM NaCl, 20 mM HEPES, pH 8, 1 mM EDTA and 0.05% DDM, in the case of solubilized membranes). The column is washed with 5 column volume of wash buffer (150 mM NaCl, 20 mM HEPES, pH 8, 1 mM EDTA and 0.05% DDM, in the case of solubilized membranes). The strep-tagged recombinant proteins are eluted with 3 column volume of elution buffer (wash buffer containing 5 mM desthiobiotin).

In both affinity chromatography techniques, the eluted proteins are concentrated using Amicons Ultra Centrifugal Filters (Invitrogen) in final volume of 500 µL (cut-of (MWCO) of each amicon depends on the molecular weight of each recompinant protein).

2.14 Ion-exchange chromatography

Ion exchange (IEX) chromatography is a technique that is commonly used in biomolecule purification. It involves the separation of molecules on the basis of their

charge. This technique exploits the interaction between charged molecules in a sample and oppositely charged moieties in the stationery phase of the chromatography matrix. Two types of ion exchange separation are possible-cation exchange and anion exchange. In anion exchange the stationary phase is positively charged whilst in cation exchange it is negatively charged.

In this study, anion-exchange chromatography was used as a second purification step, after affinity chromatography and only for the purification of IcmK. Briefly, 1 mL of Q sepharose column is equilibrated with 10 column volume of wash buffer (150 mM NaCl, 20 mM HEPES, pH 8, 1mM EDTA) and washed with 15 column volume of the same buffer. Elution takes place with 15 column volume of 1 M NaCl (Elution Buffer). As IcmK was found in the flow through, this fraction was collected and concentrated in a final volume of 500 μL, using a 50 kDa cut-off Amicon (Invitrogen).

2.15 Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC), also known as gel filtration, is one of the chromatography techniques. SEC separates molecules by differences in size as they pass through a resin packed in a column. SEC resins consist of a porous matrix of spherical particles (beads) that lack reactivity and adsorptive properties. After sample has entered the column, molecules larger than the pores are unable to diffuse into the beads, so they elute first. Molecules that range in size between the very big and very small can penetrate the pores to varying degrees based on their size.

Size Exclusion Chromatography is performed via a Superdex 200 10/300 GL column in an ÄKTA Purifier System. Superdex 200 (10–600 kDa protein resolution) is produced by the covalent bonding of dextran to highly cross-linked porous agarose beads. The separation properties of the composite medium is predominantly determined by the dextran component. Colum’s total volume is 24 mL.

All buffers must be degased and filtered before use. Initially, loop and all pumps are washed first with water and then with the buffer (150 mM NaCl, 20 mM HEPES, pH 8). Water (first) and buffer (second) wash the column and the whole system with 2 column volumes with a flow rate of 0.5 mL/min. Protein eluate from affinity chromatography is being filtered before entering ÄKTA Purifier System, in a 0,22 μm

centrifuge tube filters with centrifuge at 4^{o}C, 18.406x*g* for 10 minutes. The sample is being injected in 1 mL loop with a 1mL syringe and in absence of air bubbles. System pressure is up to 1.5 MPa and elution flow rate at 0.5 mL/min. Elution takes place with 1.5 column volume of buffer. Protein is detected at 280 nm. Finally, all fractions are collected and alalysed with SDS-PAGE (paragraph 2.9) not only for the existence of the protein, but also for its purity. Fractions which have the protein in the most pure form, are being concentrated for further use.

2.16 Differential Scanning Fluorimetry (DSF)

DSF measurements are performed as described by Boland et al. in 2018 on a Prometheus NT.48 (NanoTemper Technologies) following the manufacturer's instructions at 1°C/min from 20°C to 90°C. Cooling thermograms were recorded at 1°C/min immediately after the high temperature limited was reached. The samples were measured in quartz capillaries (Monolith NT. Label Free Zero Background MST Premium Coated Capillary) using a Monolith NT (NanoTemperTechnologies). Fluorescence measurements were made at a wavelength of 280 nm. Emitted intensities at 330 nm (F330) and 350 nm (F350) were used to calculate the DSF signal-the ratio F350/F330.

2.17 Dynamic Light Scattering (DLS)

DLS analyses were carried out using a the SpectroLight 600 (XtalConcepts GmbH, Hamburg, Germany). Samples were pipetted onto a 72-well Terasaki plate (Nunclon Delta, Nunc GmbH, Wiesbaden, Germany) in volumes of 2 mL. Prior to use, the plates were filled with paraffin oil (paraffin oil light, AppliChem, Darmstadt, Germany) to protect the sample solutions from drying out. The laser wavelength used was 660 nm at a power of 100 mW. The scattering angle for placement of the detector was fixed at 150. All samples were measured at 293 K.

2.18 Circular Dichroism spectrospory

All the experiments are performed at 25°C using a Jasco CD Spectrometer (J-810). Briefly, for all the measurements potassium phosphate (KPi) buffer (20 mM KPi, pH 7.5, 150 mM NaCl, 0,02% DDM or 0,1% LDAO or 0,05% Foscholin-14 or 0,1% Foscholin-12 respectively) was used and all Far- UV CD spectra were recorded from 190-260 nm. Different protein concentrations are being examined, in order to find the

the most appropriate for the measurements (In the current study, 3 μM was the best concentration for both proteins of interest). Additionally, a demountable rectangular Quartz SUPRASIL (Hellma®) cuvette of 0,1 cm light-path length is used for the experiments. The scanning speed was 20 nm/min with a scan time per point of 1 s, data pitch of 0.5 nm and band width of 1 nm. Ten repeat scans per sample were made (accumulation: 10), then averaged and smoothed in Jasco software. Each averaged CD spectrum was corrected for the buffer baseline by subtracting an averaged buffer CD signal over the same wavelength region [Ioannou et al, 2015]. Finally, the resulting ellipticity data (millidegrees) were converted into mean molar ellipticity ([Θ], $deg*cm^2*dmol^{-1}$). For the data processing, origin 2020 software was used and for structure prediction, the on-line tool “BeStSeL” (Beta Structure Selection).

2.19 ELISA Assay Indirect

ELISA method was used in this work. In this type of ELISA, the antigen of choice is bound to the experimental surface, and an unlabeled primary antibody specific for it is added and binds to the antigen. Subsequently, an enzyme-labeled secondary antibody is added and binds to the primary antibody. A colorless substrate is introduced to the sample, which reacts with the enzyme conjugate, and produces a measurable byproduct. Depending on the choice of substrate, this byproduct can be either colourimetric, chemiluminescent or fluorescent.

Microtiter flat bottom plates (96 wells, Corning Costar) are coated with different concentrations of purified recombenant proteins (1.0, 0.5, 0.25, 0.125, 0.0625, 0.03 and 0.015 μg/ml) diluted in carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. The wells were then washed with PBS-T washing buffer (137 mM NaCl, 2.7 mM KCl, 0.01 M Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween 20, pH 7.4)and blocked with blocking buffer (PBS-T and 1%(w/v) bovine serum albumin (BSA) at 37°C for 60 minutes. After blocking, the wells were incubated with a solution of Q fever positive serum (1:25 to 1:6400 serially diluted in blocking buffer) for 60 minutes at 37°C, washed three times with washing buffer, and incubated with peroxidase conjugated rabbit anti-human IgG (1:3000 diluted in blocking buffer, Boster Biological Technology) at 37°C for 1 hour. Following three washing cycles, the wells were incubated with a solution of substrate/chromogen (TMB Core Biorad) for 10 minutes

and the colourimetric reaction was stopped by adding 0.5 M H_2SO_4. All reagents were added at a volume of 100 μl/well. The optical density was read at 450 nm on a microplate reader (Multiskan FC, Thermo Fisher Scientific).

The optimal concentration for the antigen was determined by the lowest concentration which demonstrated the positivity of the reaction at any dilution of the positive serum. The optimal dilution of the serum was presented by the greatest difference in reading between the positive and negative serum in the optimal antigen concentration.

2.20 Samples sera

In this study, a total of 160 patients' samples sera was used. All sample sera provided by the sera collection of the Hellenic National reference center for Q fever (Crete, Greece). In addition, all patients were asked to fill in a consent form concerning future research studies utilizing their samples. Briefly, patients were divided into five groups:

a) people suffering from persistent focalized *C.burnetii* infection

b) healthy blood donors (as a control group)

c) people suffering from acute Q fever

d) people suffering from acute Q fever but at least one sample of each patient was tested ≥1/1024 IgG phase I

e) people suffering from rheumatoid disease (to test potential cross reactions)

2.21 Statistical analysis

Statistical Analysis is a very useful mathematic tool that allows scientist to analyze their data and find an application in every day life. In this research, statistical analysis was used in order to have our results visualized and finally answer the question if the expressed proteins act as Q fever differential diagnostic tools.

So far, we have managed to proceed to statistical analysis only for targets 1, 2, 3, 4, 6 (Table 1).

To perform the statistical analysis (IBM SPSS Statistics v.25), P values <0.05 are considered to be statistically significant. For each protein, a receiver operating characteristic (ROC) curve analysis is used; following the area under curve (AUC) estimation, the cut-off value maximizing the Youden's index (J=sensitivity+specificity-1) is selected as optimal cut-off value. Each protein is considered as positive for the new diagnostic test (ELISA) following its classification to the two categories: 0="no disease" or 1="disease", by considering each value lower than the cutoff as 0 (no disease) and each value larger or equal to the cutoff as 1 (disease). In the continuity, the corresponding classification 2x2 table of each protein is obtained in reference to the standard clinical classification. The values of true positives, true negatives, false positives and false negatives of that table are used for the calculation of a series of indicators of the agreement between the classification obtained by the protein and the existing clinical classification, that is indicators of the performance of the protein as a possible diagnostic test: sensitivity, specificity, positive predictive value, negative predictive value and Cohen's kappa coefficient (κ).

Apart from the above approach we, also, processed the protein values using Binary logistic regression analysis to find any possible combinations of proteins that could act as better diagnostic factors. The above-mentioned software was again used.

3. RESULTS

3.1 Cloning of the genes of interest

In the current study, 18 proteins are selected to be validated of their antigenic properties in order to differentiate the chronic from acute infections of Q fever. More specific, six of them are described in literature as chronic Q fever specific, nine are described as acute Q fever specific and three of them belong to the secretion system type IVB of *Coxiella burnetii*. For the heterologous expression of the antigenic proteins in *E. coli*, full-length genes were amplified from *C. burnetii* genomic DNA. These successfully amplified genes are CBU_1910 (Com1), CBU_1718 (HspB), CBU_0092 (YbgF), CBU_0937 (UPF04220), CBU_0612 (OmpH), CBU_0271 (ssb), CBU_0952 , CBU_0891, CBU_1143 (yazC), CBU_0545 (LemA),CBU_1398 (sucB), CBU_1626 (IcmG), CBU_1628 (IcmK), CBU_1644 (DotC) and are demonstrated in Figure 7. The obtained size correlated with the calculated mass for each gene respectively.

The ampliefied genes were subsequently cloned into the NdeI/XhoI sites of pET-22b(+), resulting in expression vector with C-terminal His6-tag and N-terminal native signal peptide, if any is present. Among eighteen targets, we were able to clone fourteen genes into pET-22b(+) vector (Figure 7). All plasmids constructed are provided in the Supplementary Materials (Figure S1).

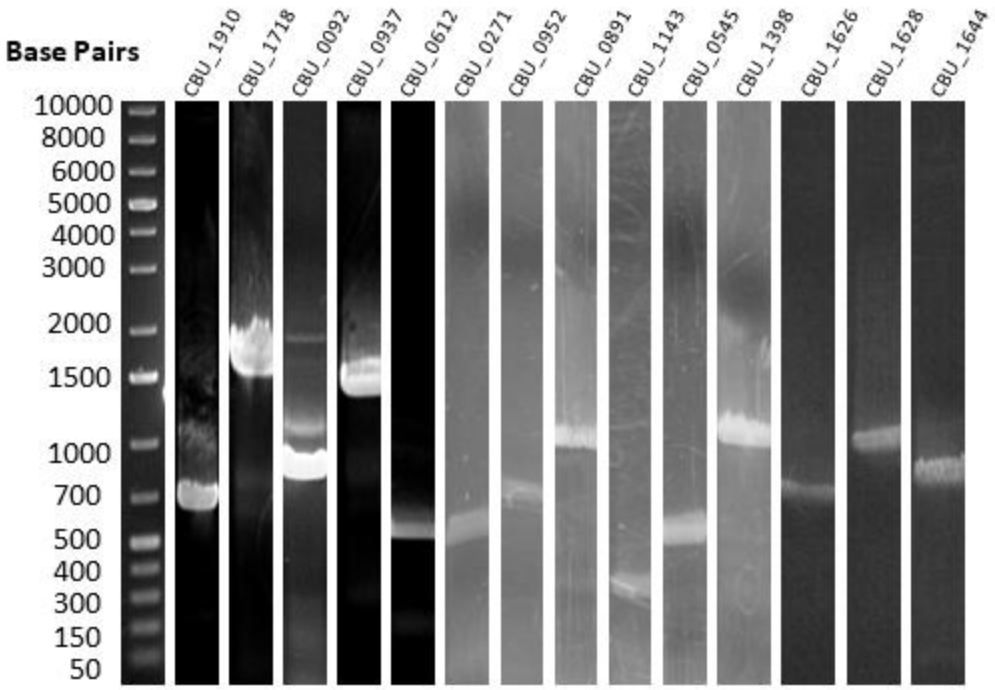

Figure 7: Agarose gel showing all fragments that have been amplified and their sizes.

3.2 Expression of the antigenic proteins After constructing the expression vectors, they were transformed into *E. coli* BL21 (DE3) cells. For targets 1-6 (Table 1) small-scale (50 mL of culture) expression trials were performed by varying growth temperatures, induction time and concentrations of inducer. More specifically, transformed *E. coli* cells were cultured in two different temperatures (37^{o}C and 32^{o}C) and induced with two different concentrations of IPTG (0.5 mM and 1 mM). After taking 1 mL of cell culture sample before induction and after induction in three different time-points (2 h, 4 h, overnight), conclusions were made about the best expressing conditions of each protein. So, it was shown that all proteins (apart from CBU_0937) are best expressed in 37^{o}C, with 1 mM of IPTG and culture four hours after induction (Figure 8).

However, under the conditions tested, no apparent expression was observed for CBU_0937, while the expression of the other proteins was detected by immunodetection (pa. 2.10). In order to obtain the recombinant protein of CBU_0937, its predicted signal sequence was removed and the region encoding the mature protein was fused to the pelB leader sequence. By employing the pelB leader

sequence for periplasmic localization in *E. coli*, significant expression of CBU_0937 was achieved. Finally, CBU_0937 was expressed in 1mM of IPTG giving its best signal 4h after induction (Figure 8).

For targets 7-15 (Table 1) no expression test took place. Instead, these recombinant proteins seemed to be expressed at 37^{o}C with 1 mM of IPTG and 4 h after induction (Figure 8).

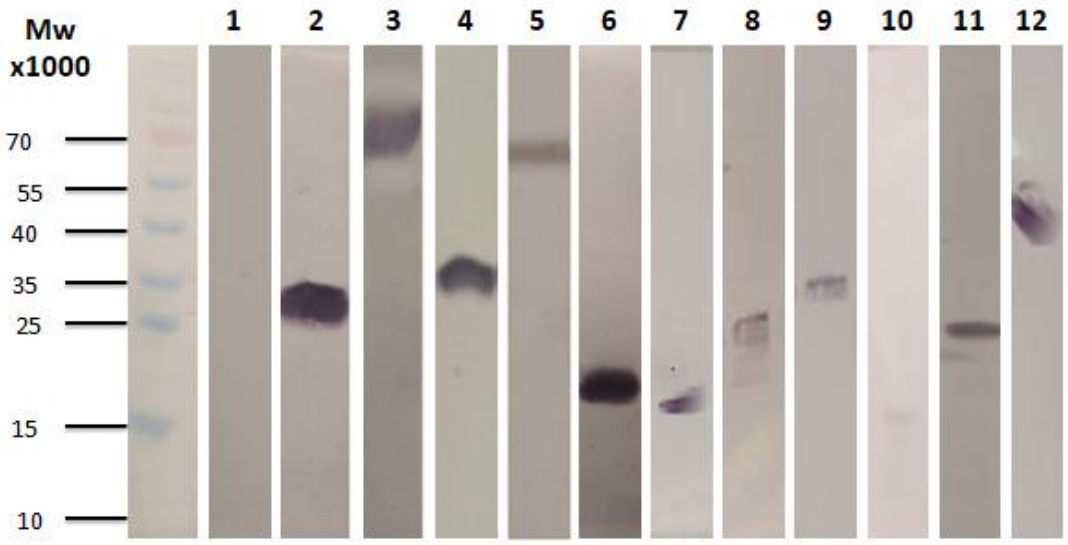

Figure 8: Immunoblotting of the proteins after expression test. Anti-polyHistidine- Alkaline Phosphate antibody was used to detect the recombinant proteins. Lanes 2-12 correspond to samples taken 4 hours after induction with 1mM IPTG, for the following targets: CBU_1910, CBU_1718, CBU_0092, CBU_0937, CBU_0612, CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545 AND CBU_1398 respectively. Lane 1 is used as control, representing a sample taken before the induction.

As it was mentioned above, CBU_1626 (IcmG), CBU_1628 (IcmK) and CBU_1644 (DotC) are three proteins of the secretion system type IV of *Coxiella burnetii*, that were tested for their ability to diagnose acute or chronic (or both) Q fever.

In this study, we used plasmids pTTQ18- G-C-strep, pTTQ18- K-C-strep and pTTQ18- C3-C and *E. coli* as host. The first two vectors curry the fusing 8aa-StrepII-tag (at the C-terminus of each protein) as a recognition site while pTTQ18-C3-C curries both His-tag (at the N-terminal of the protein) and 8aa-StrepII-tag (at the C-terminal of the protein). Similar to antigenic proteins, an expression test was also

performed, in order to detect the best expression conditions for these proteins as well.

Figure 9 shows the best conditions after examination of different IPTG concentration and different temperatures. We found that only in the case of IcmK low IPTG concentration (0,5 mM), low temperature (20^{o}C) and long incubation time after induction (overnight) increased the expression of the recombinant protein, while 37^{o}C, 1 mM of IPTG and incubation for 3 hours after induction were the best expression conditions for IcmG and DotC.

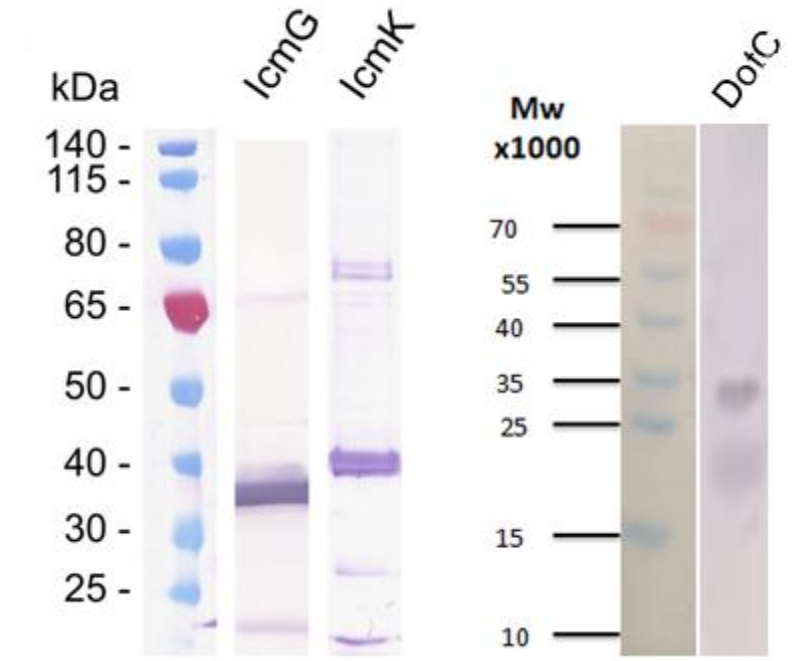

Figure 9: Western Blot showing the best expression conditions for IcmG, IcmK and DotC. IcmG and DotC are best expressed with 1 mM of IPTG 3 h after induction, while IcmK seems to be best expressed with 0.5 mM IPTG and incubation overnight at 20°C.

Table 2 presents the expression details for all targets.

Table 2: Proteins of our interest and their best expression conditions.

Number	Gene Name	Protein Name	Successful clonning	Expression conditions		
				Temperature (^{o}C) after induction	IPTG concentration (mM)	Incubation time (h) after induction
1	Com1	CBU_1910	Yes	37	1	4
2	HspB (GroEl)	CBU_1718	Yes	37	1	4
3	YbgF	CBU_0092	Yes	37	1	4
4	UPF0422	CBU_0937	Yes	37	1	4
5	Tuf-2	CBU_0236	No			
6	OmpH	CBU_0612	Yes	37	1	4
7	ssb	CBU_0271	Yes	37	1	4
8	-	CBU_0632	No			
9	-	CBU_0952	Yes	37	1	4
10	Omp1	CBU_0311	No			
11	rplL	CBU_0229	No			
12	-	CBU_0891	Yes	37	1	4
13	yazC	CBU_1143	Yes	37	1	4
14	LemA	CBU_0545	Yes	37	1	4
15	sucB	CBU_1398	Yes	37	1	4
16	IcmG	CBU_1626	Yes	37	1	3
17	IcmK	CBU_1628	Yes	20	0.5	Overnight
18	DotC	CBU_1644	Yes	37	1	3

3.3 Purification of the recombinant proteins using Ni-NTA affinity chromatography

After finding the best expression conditions, larger scale culture (1 L) was performed. Cells were harvested 4 h after induction and broken by the French Press (Pa. 2.6). After ultracentrifugation, membrane and soluble fractions were collected separately. For those proteins whose localization is predicted (Table 1), the corresponding fraction (membrane or soluble) was kept for further study. More specifically, the membrane fraction was kept for membrane proteins (CBU_1910, CBU_0937, CBU_0612, CBU_0891, CBU_1143, CBU_0545) and the soluble fraction for the proteins that are predicted to be localized at the periplasm (CBU_1718, CBU_0092) (Table 1).

CBU_0271, CBU_0952 and CBU_1398 are three proteins whose localization inside the cell hasn't been predicted yet. For this reason, immunobloting was performed in both soluble and membrane part, in order to find out where the proteins are located. In Figure 10, we can see that all three proteins are located in the membranes.

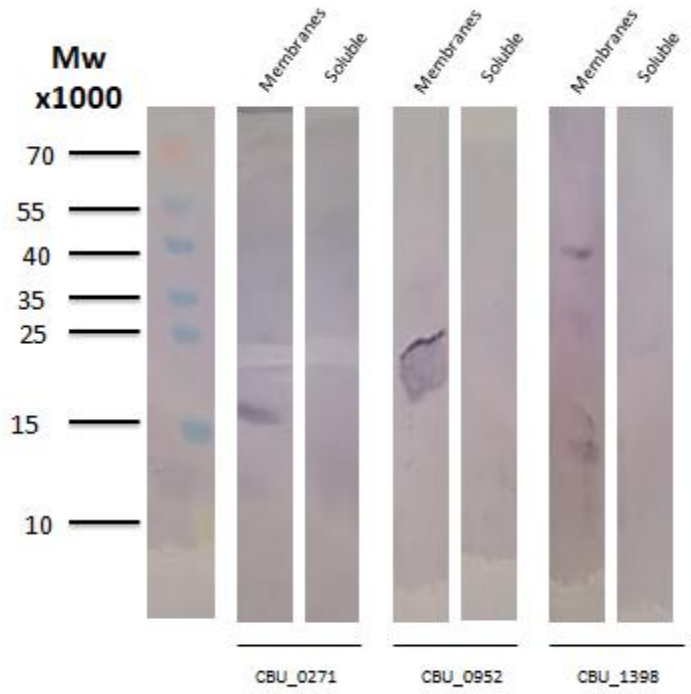

Figure 10: Western Blot showing in which fraction CBU_0271, CBU_0952 and CBU_1398 are detectable.

In the case of membrane proteins, membranes were first solubilized using DDM in final concentration of 1%(w/v) (Pa. 2.12). All recombinant proteins were purified using Ni-NTA affinity chromatography, as described in paragraph 2.13. SDS- PAGE was then performed in order to check the purity of each protein. The Coomassie-stained SDS-PAGE gels demonstrated that targets 1-4 and 6 (Table 1) can be purified to relatively high purity in one affinity purification step (Figure 11). In addition, the apparent molecular masses observed for the purified proteins in SDS-PAGE is in agreement with the calculated masses of the respective proteins.

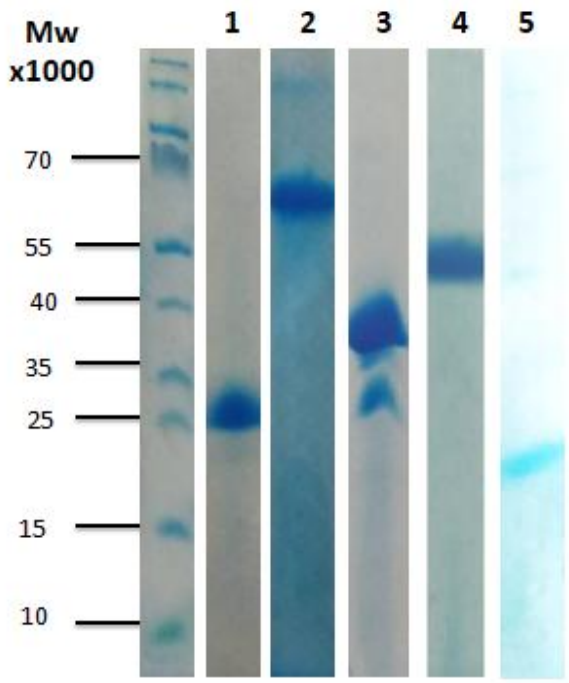

Figure 11: SDS- PAGE Coomasie stained gel for purified recombinant proteins. Lane 1 corresponds to CBU_1910, lane 2 to CBU_1718, lane 3 to CBU_0092, lane 4 to CBU_0937 and lane 5 corresponds to CBU_0612.

Regarding to targets 7, 9, 12-15 (Table 1), one purification step (affinity chromatography) wasn't enough to isolate the proteins in high purity. In addition, the yield wasn't that high, however it was adequate for further test, such as Western blot and ELISA (Pa. 2.19).

3.4 Purification of recombinant proteins using Streptactin affinity chromatography and Gel Filtration

For IcmG and IcmK, large scale culture (12L) was performed and the cells were disrupted using a Microfluodizer (Pa. 2.6). Afterwards, the membrane and the soluble part were collected separately. The localization of the expressed targets was tested by immune detection of the StrepII-tag. It has been shown in the case of *Legionella* pneumophila that DotF (IcmG) is an inner membrane protein and the DotH (IcmK) an outer membrane protein [Vincent et al, 2006]. While IcmG-StrepII was detected only in the membrane fraction, we detected the IcmK-StrepII in the soluble fraction with a weaker signal in the membrane fraction.

Proceeding further, in case of IcmG, the efficiency of different detergents (nonionic or zwitter ionic) was investigated. Collected membranes were solubilized with 1% (w/v) of dodecyl-maltoside, LDAO, LDAO/DDM, octyl glucoside and Triton. After application of the affinity purification step in the presence of used solubilization detergents, analytical gel filtration was applied in order to investigate the homogeneity of the isolated protein. The best results concerning solubilization efficiency and purity of the isolated protein was achieved by LDAO (Figure S2). For this reason we used LDAO for the solubilization and purification of the recombinant IcmG. Figure 12 shows the purified protein after affinity chromatography and gel filtration. As the elution profile shows the isolated protein is homogenous.

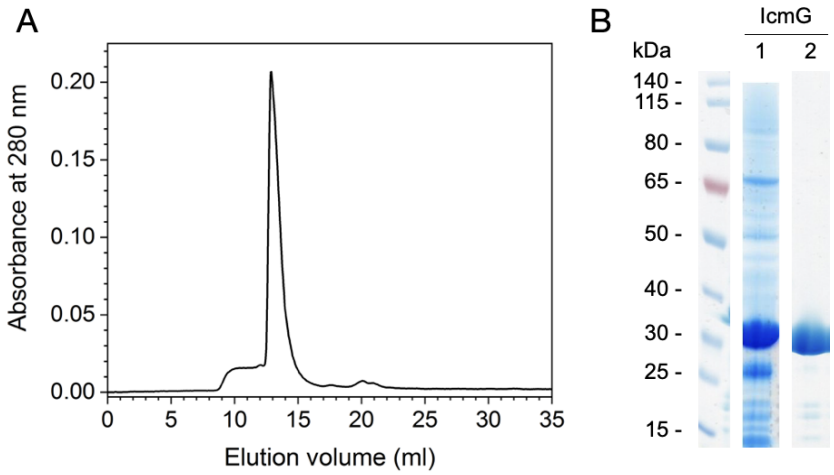

Figure 12: Purification of IcmG-StrepII. A) Gel Filtration elution profile detected at 280 nm. B) Lane 1, IcmG-StrepII after affinity chromatography. Lane 2, main peak of the gel fitration.

In regard to IcmK-StrepII, the first purification attempt using Strep-Tachin agarose affinity chromatograph with a subsequent size exclusion chromatography allowed the purification of the protein from the supernatant of whole cell lysate with a high purity. An anion exchange chromatography was used as additional purification step after affinity chromatography in order to isolated IcmK in much higher purity (Figure 13).

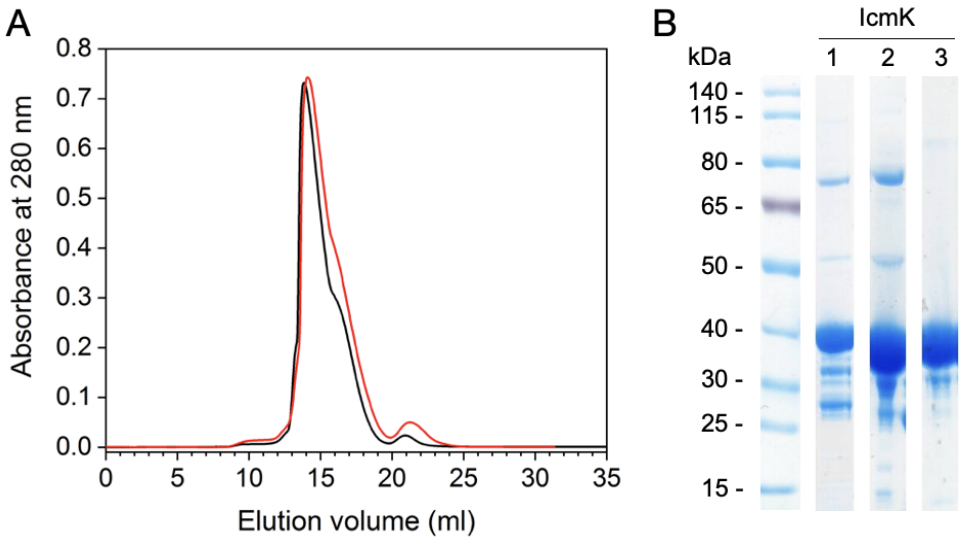

Figure 13: Purification of the IcmK-StrepII. A) Gel filtration elution profile detected at 280 nm. Red line without the Q-Sepharose step. Black line after Q-Sepharose. B) SDS=PAGE coomasie stained gel. Lane 1, IcmK-StrepII after affinity chromatography. Lane 2, main peak of the gel fitration without the Q-Sepharose purification step. Lane 3, main peak of the gel fitration after a Q-Sepharose purification step.

In the case of DotC, *E. coli* cells were cultured in 1 L of LB and after harvesting, the cells were disrupted by French press. Subsequently, the membrane and the soluble fraction were collected and protein was detected in the membranes by Western blot. DotC was isolated and purified by Ni-NTA affinity chromatography. SDS-PAGE Coomasie stained gel indicates that DotC can be isolated and purified in one purification step (Figure 14).

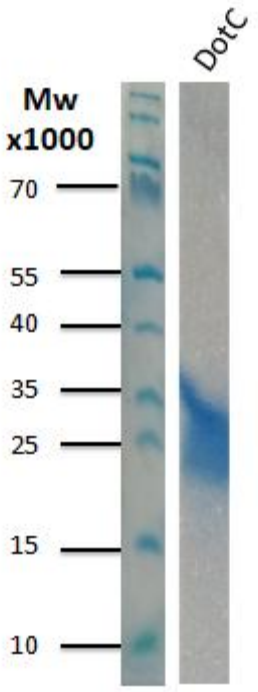

Figure 14: SDS- PAGE coomassie stained gel. DotC seems pure enough after affinity chromatography purification.

3.5 Study of the biophysical properties of IcmG and IcmK.

IcmG and IcmK are two proteins belonging to the T4BSS of *Coxiella burnetii.* In addition, along with IcmE, DotC and DotD, they are essential for the formation of the Dot/Icm core complex. For this reason, isolation of these proteins in great yields and further study of their structure will support the attempts for the development of drugs or vaccines against *Coxiella*.

As it was mentioned above, we were able to isolate IcmG and IcmK in sufficient amounts (1.2 mg/L and 3 mg/L of bacterial culture respectively) and high purity. This gave us the opportunity to use the proteins for subsequent structural analysis. In order to increase the change for the crystallization of these targets biochemical and biophysical analyses was perfomed.

3.5.1 Oligomer state

Structural information about the T4SS of *A. tumefaciens* and *L. pneumophila* demonstrated that IcmK and IcmG structure oligomers in the native secretion system [Kubori et al, 2014]. So, in order to examine the oligomer condition of the recombinant proteins, recombinant IcmG and IcmK were analyzed by BN-PAGE

(Pa. 2.11). In addition, the oligomer state of IcmG was determined in four different detergents (DDM, LDAO, Fos-Cholin 12 and Fos-Cholin 14). Figure 15 illustrates the results of the corresponding BN-PAGE gel.

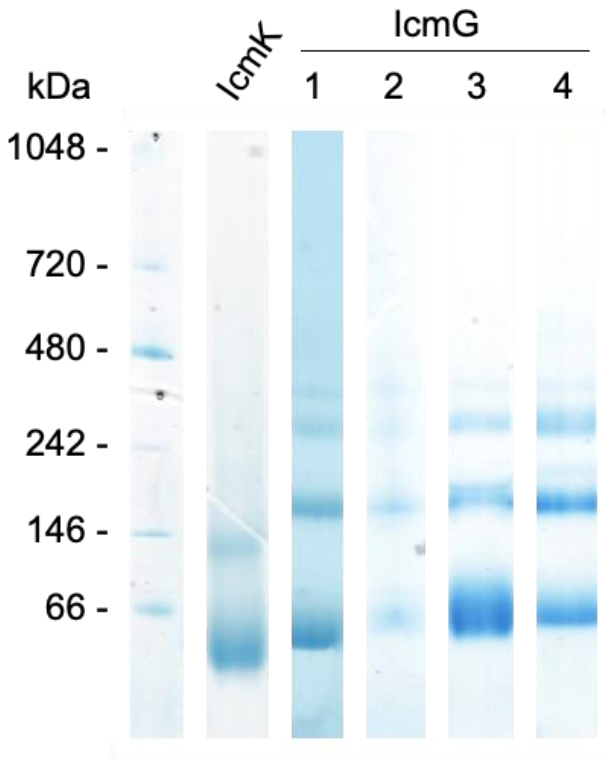

Figure 15: BN-PAGE gel presenting the oligomer state of the two proteins. IcmG was analyzed in four different detergents. Lane 1 corresponds to LDAO, lane 2 to DDM, lane 3 to Fos-Cholin 12 and lane 4 corresponds to Fos-Cholin 14.

According to BN-PAGE gel (Figure 15), in the case of IcmK a main band at approximately 50 KDa and one minor band at about 140 KDa were detected, indicating the presence mainly of monomers. On the other hand, in the case of IcmG purified with LDAO, several protein bands with different molecular masses can be observed (Figure 15). The main bands are present at 66 and 150 KDa, suggesting the presence of monomers and dimers of the IcmG. In addition, the presence of additional bands at higher molecular weight between 300 and 400 KDa may also indicate the formation of high oligomeric complex of IcmG. Concerning the oligomer state of IcmG in DDM, Fos-Cholin 12 and Fos-Cholin 14, there is no significant difference in its pattern.

3.5.2 Characterization of IcmG and IcmK by DLS, far -UV CD and DSF

In further analysis, the hydrodynamic dimension of the purified recombinant proteins, their secondary structure and their purification and storage conditions were evaluated by DLS (Pa. 2.17), far-UV CD (Pa. 2.18) and DSF (Pa. 2.16) respectively. In the case of IcmG, all these properties were tested in four different detergents, as it was mentioned above. Figures 16-21 present the results of these tests. DSF thermostability analysis for IcmG in LDAO, in Fos-Cholin 12 and in Fos-Cholin 14 can be found in the supplementary materials.

Dynamic light scattering (DLS) is a technique that can be used for measuring the size and size distribution of molecules and particles typically in the submicron region. Typical applications of dynamic light scattering are the characterization of particles or molecules which have been dispersed or dissolved in a liquid [Meyer et al, 2014].

DLS technique was used in this study in order to test the biophysical properties of the membrane protein IcmG in four different detergents as well as the soluble protein IcmK.

To start with, in the case of IcmK, the particle size of the protein was determined to be 3.9±0.06 nm, suggesting that IcmK may exist in a monomeric form (Figure 16).

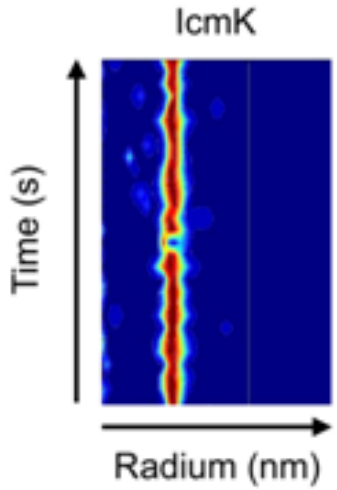

Figure 16: Differential light scattering measurements of purified IcmK. Radius distribution plot of protein particles were shown.

Concerning IcmG, which is a membrane protein, all tests mentioned above were performed in the presence of the four different detergents (LDAO, DDM, Fos-Cholin 12, Fos-Cholin 14). According to DLS results (Figure 17), the average particle size of IcmG varies from 3.9 nm for LDAO to 7.5 nm for DDM. The size of IcmG in Fos-Cholin 14 is close to that observed for LDAO solubilized samples, while the size of IcmG in Fos-Cholin 12 is about 4.4 nm.

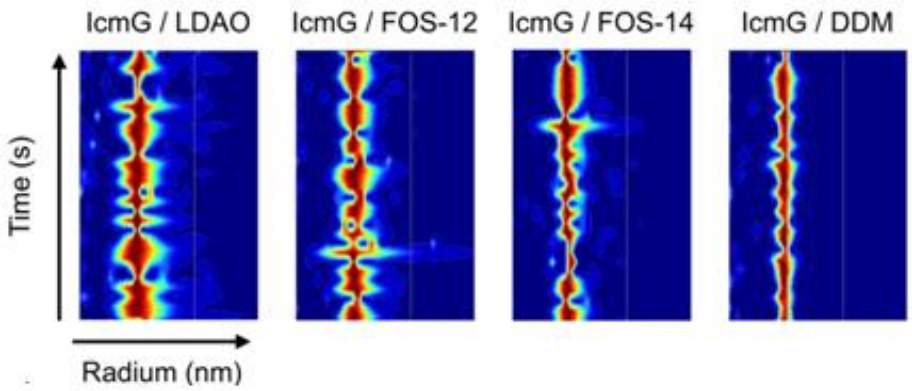

Figure 17: Differential light scattering measurements of purified IcmG in the presence of different detergents. Radius distribution plot of protein particles were shown.

Circular Dichroism (CD) is a very useful tool for the determination of the secondary structure and folding properties of proteins that have been obtained using recombinant techniques or purified from tissues. CD is an absorption spectroscopy method based on the differential absorption of left and right circularly polarized light. Optically active chiral molecules will preferentially absorb one direction of the circularly polarized light. The difference in absorption of the left and right circularly polarized light can be measured and quantified. It has been shown that CD spectra between 260 and approximately 180 nm can be analyzed for the different secondary structural types: alpha helix, parallel and antiparallel beta sheet, turn, and other [Greenfield, 2006].

The CD spectrum of IcmK exhibits one minimum at 205 nm and a small shoulder at 220 nm indicating the presence of α-helical structure (Figure 18). It was predicted that IcmK contains 30% β-sheet, 10% α-helices, 13% turns and 50% others demonstrating the flexibility of this protein [Greenfield et al, 2006].

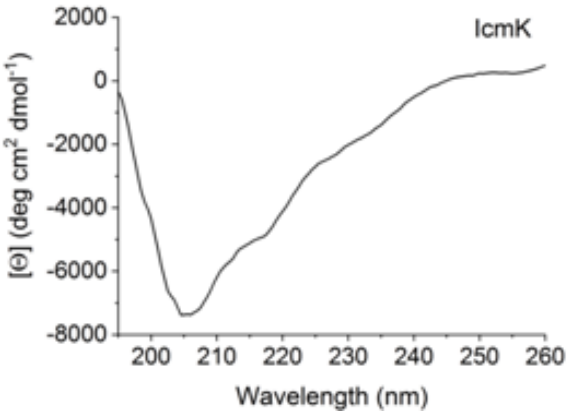

Figure 18: Circular dichroism spectra of IcmK.

CD spectroscopy was applied in order to characterize the secondary structure of IcmG (Figure 19). The CD spectra of IcmG show similarity to that of IcmK. It was predicted that IcmG contains 21% β-sheet, 18% α-helices, 15% turns and 44% others demonstrating the less flexible structure of this protein compare to the IcmK [Greenfield et al, 2006]. In addition, the CD spectra showed that the detergents slightly influence the secondary structure.

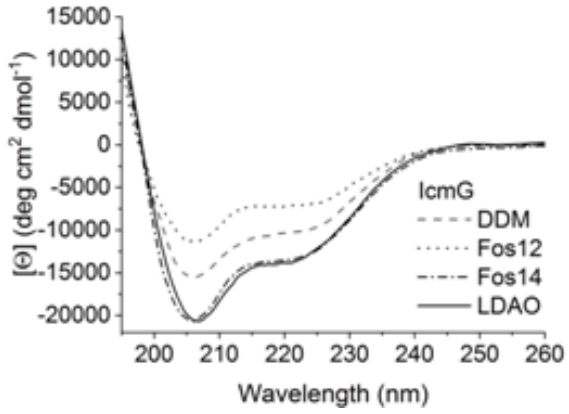

Figure 19: Circular dichroism spectra of IcmG.

Differential Scanning Fluorimetry (DSF) is a high throughput screening method for protein solutions. Also called the thermofluor assay or thermal shift assay, DSF is a method that follows the refolding of a protein as it experiences progressive denaturation. The technique relies on using specific dyes that fluoresce as they

interact with proteins in their denaturation transition between a strongly hydrated solvated state and the final aggregated solid state [Vollrath et al, 2014].

DSF screens are designed to evaluate purification and storage conditions. According to Figure 20, IcmK exhibits a melting point (T_m) of about 56°C. This information means that the protein remains stable at both 4°C and room temperature, making its handling easier.

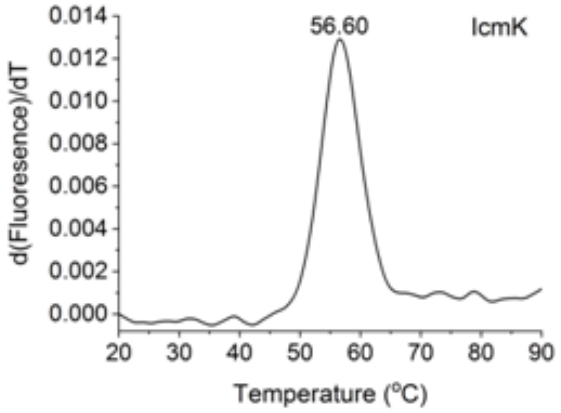

Figure 20: Analysis of thermostability of IcmK by nano differential scanning fluorimetry. Transition temperature is indicated.

In Figure 21 we can see the melting point of the IcmG protein in DDM. The T_m in the four different detergents varies from 45°C for Fos-Cholin 14 to 55°C for LDAO (Figure S3). Although highest T_m was observed for LDAO, only in DDM the protein has showed a sharp peak, indicating that in this detergent IcmG is more stable.

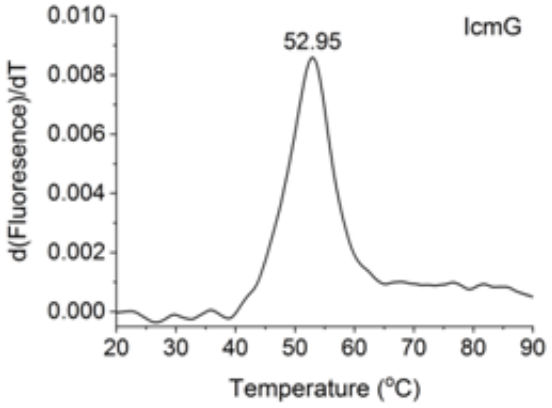

Figure 21: Analysis of thermostability of IcmG by nano differential scanning fluorimetry. Transition temperatures are indicated.

3.6 Study all recombinant proteins about their ability to differential diagnose Q fever.

All recombinant proteins that were expressed, isolated and purified were tested for their diagnostic ability in chronic or acute Q fever in patients serum samples (Table S5), firstly by western blot. At this point we should mention that sample sera used in this study were provided by the sera collection of the Hellenic National reference center for Q fever (Crete, Greece). In addition, all patients were asked to fill in a consent form concerning future research studies utilizing their samples.

3.6.1 Immunodetection of targets specific in the chronic form of Q fever.

Five recombinant proteins (CBU_1910, CBU_1718, CBU_0092, CBU_0937 and CBU_0612) are predicted to be specific and sensitive in diagnosis of chronic Q fever (Table 1). As it was mentioned above, they were expressed and isolated in adequate amounts (1,4 mg/L of bacterial culture) and purified in one purification step resulting to relative high purity.

Afterwards, the proteins were tested by western blot, using 4 different sera samples from patients suffering from chronic Q fever (immunofluorescence assay (IFA) result: immunoglobulin G(IgG) > 4096 and endocarditis) and 4 different sera samples from patients suffering from acute Q fever in 4 different experiments. Figure 22 shows one representative immunoblot result. It is clear that all proteins can be detected using the serum collected from the patient suffering from chronic Q fever, while no immunoreaction was observed when the serum from patient with acute Q fever was used.

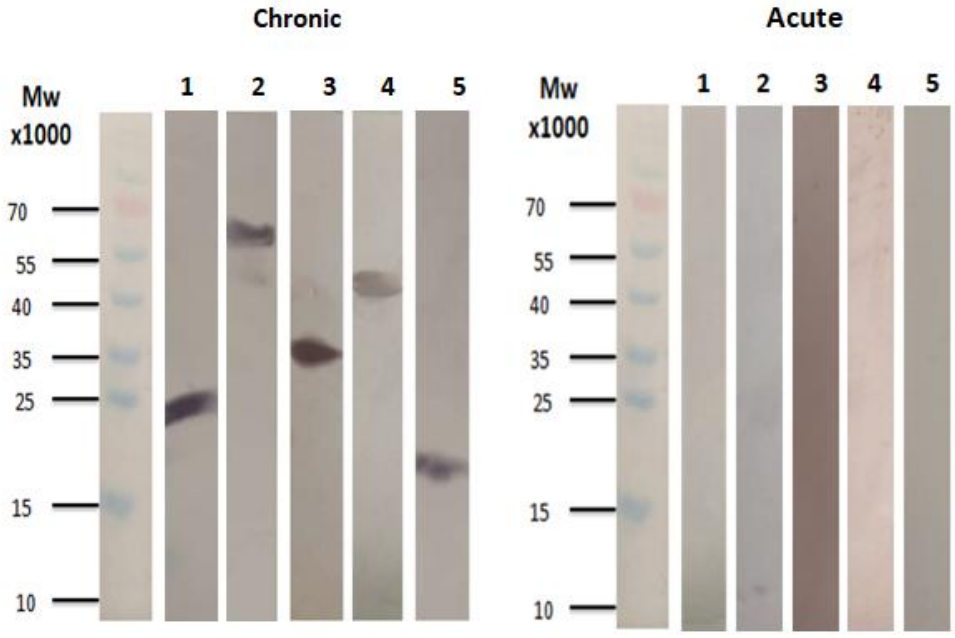

Figure 22: Immunoblotting using sera from patients with chronic Q fever and sera from patients with acute Q fever. 1. Corresponds to CBU_1910 2. Corresponds to CBU_1718, 3. Corresponds to CBU_0092, 4. Corresponds to CBU_0937, and 5. Corresponds to CBU_0612.

3.6.2 Immunodetection of targets specific in the acute form of Q fever.

According to literature [Kowalczewska et al, 2011], CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545 and CBU_1398 have been described as specific and sensitive in acute Q fever. We have already mentioned in our preliminary results we tried only one expression condition for these targets. In addition, we weren't able to isolate them in high yields (0,3 mg/L of bacterial culture) and high purity after one step of purification. However, the recombinant proteins were detected in immynoblot by a monoclonal anti-poly-histidine alkaline phosphatase conjugated antibody not only after expression (Figure 7) but also after purification (Figure S4). This is why we decided to test those targets with the patient sera samples, as well. In this case we tried 2 different sera samples from patients suffering from chronic Q fever and 2 different sera samples from patients suffering from the acute type of disease. In figure 23 we can see the corresponding western blot,that indicates the reaction of almost all the proteins in both acute and chronic Q fever. In fact, only CBU_1398 seems to react with sera from patients with chronic Q fever while it doesn't give any signal with the sera from patient suffering from the acute Q fever (Figure 23)

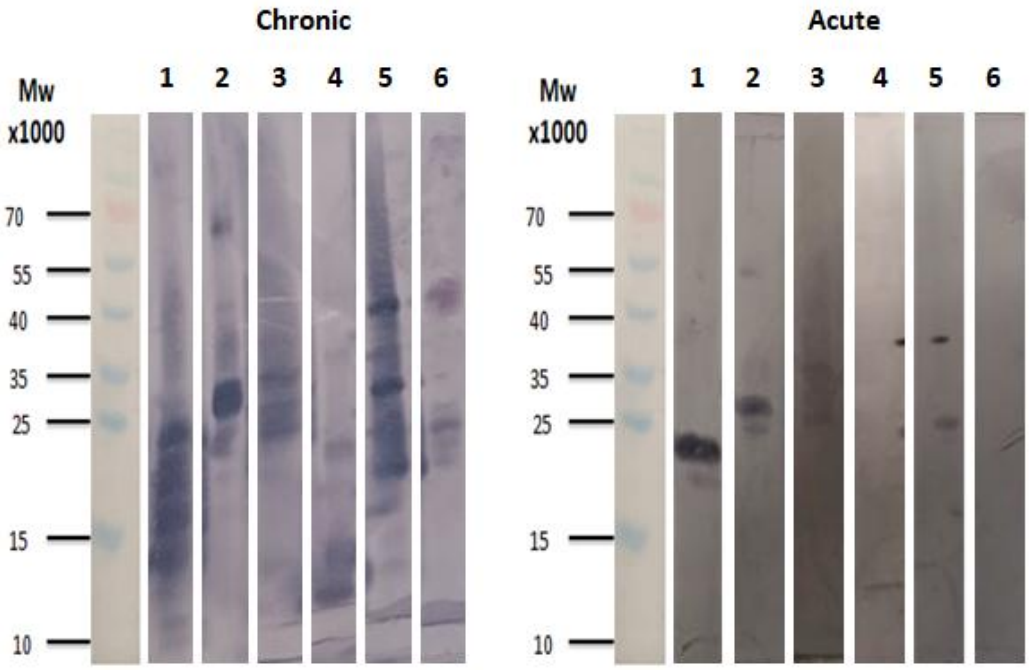

Figure 23: Immunoblotting using sera from patients with chronic Q fever and sera from patients with acute Q fever. 1. Corresponds to CBU_0271 2. Corresponds to CBU_0952, 3. Corresponds to CBU_0891, 4. Corresponds to CBU_1143, 5. Corresponds to CBU_0545 and 6. Corresponds to CBU_1398.

3.6.3 Study of the antigenicity of IcmG, IcmK and DotC

As it is well known, T4BSS of *Coxiella burnetii* plays a crucial role in the virulence of the pathogen. Testing these three Type IV secretion system proteins, we can investigate their potential function in the diagnosis of the disease.

IcmG, IcmK and DotC have been successfully expressed, isolated and purified. Three sera samples from patients suffering from chronic Q fever and three sera samples from patients with the acute type of the disease were used in order to test these proteins antigenicity. Figure 24 displays a representative imunoblotting result. As it seems, IcmG and IcmK are recognized by both the sera from patients with the chronic Q fever and the sera from patients with the acute form of the disease while DotC doesn't react with any of them.

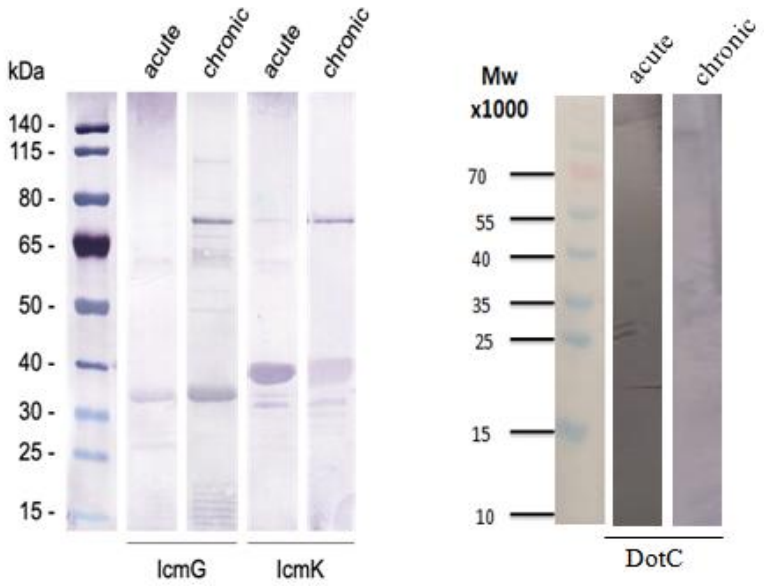

Figure 24: Immunodetection using sera from patient diagnosed with acute or chronic Q fever. IcmG, IcmK and DotC were tested.

3.7 Serum samples

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies and hormones. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction. ELISA principles are very similar to other immunoassay technologies. ELISA relies on specific antibodies to bind the target antigen and a detection system to indicate the presence and quantity of antigen.

A total of 160 sera samples were re-tested using the IFA technique (FOCUS Diagnostics, Cypress, CA, USA). Out of these 160 samples, 20 samples were from chronic patients (based both on laboratory [≥1/1024 IgG phase I] and clinical findings). The lowest observed IgG phase I titer in this group was 1/4096 (Table S5). The blood donors' group, considered as our healthy population-Q fever negative sample, consisted of 12 sample sera. All but one serum indicated lower than 1/64 titer against IgG phase I. Interestingly, one blood donor sample indicated a titer of 1/4096 positive for chronic Q fever according to IFA results (IgG phase I≥1/1024).

Since the sample serum originated from a blood donor (not a patient) we tend to believe that there is a case of false positive test result by IFA. On the other hand, Q fever may be asymptomatic. In this case the observed high titre antibodies is directed against phase I of the bacterium (indicative of chronic Q fever), which cannot be the case in a healthy individual [Vranakis et al, 2019]. Unfortunately, although it would be of particular interest to follow up this individual's serological profile in order to see if we are talking about a false chronic Q fever positive result or an asymptomatic acute Q fever case, we failed to get access to a 2nd sample, that's why we chose to exclude him from the study.

Patients with high immunoglobulin titers against *C. burnetii* IgG phase II but lower than 1024 against phase I was our next group and consisted of 61 samples. The next group of patients consisted of 47 sample sera which indicated at least one sample ≥1/1024 IgG phase I (positive laboratory diagnosis). However, the final diagnosis for this group of patients was not chronic Q fever. Finally, 20 sample sera from patients with rheumatoid disease was our last group of samples. As it is commonly known samples originating from patients with any kind of rheumatoid disease can cross react in immunofluorescence assays providing falsely positive results, three out of 20 samples indicated IgG Phase I titers higher than 1/2048 [Vranakis et al, 2019].

Table 3 summarizes the groups of patients' sample sera that were re-tested with IFA and analyzed with the under development indirect ELISA.

Table 3: Sample sera categorized according to the Q fever laboratory diagnosis and the final diagnosis (laboratory diagnosis and clinical findings).

Number of sample sera	**Clinical Diagnosis**	**Laboratory diagnosis**	
		Positive for Acute Q fever ≥1/1920 IgG total	**Positive for Chronic Q fever ≥1/1024 IgG phase I**
20	Chronic Q fever		100%
12	Blood donors		8.3% (1/12)
61	Acute Q fever	100%	0
47	Acute Q fever (at least one sample of each patient was tested ≥1/1024 IgG phase I)	100%	68.1% (32/47)
20	Rheumatoid disease	55% (11/20)	15% (3/20)

3.8 Indirect ELISA

In this study, ELISA tests can be divided in three main groups:

a) ELISA about CBU_1910, CBU_1718, CBU_0092, CBU_0937 and CBU_0612
b) ELISA about CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545, CBU_1398 and DotC
c) ELISA about IcmG, and IcmK

In the first group, all 160 different sample sera (Table 3) were used in order to test the 5 antigenic proteins. A simple statistical analysis about ELISA results was followed, allowing us to extract not only qualitative but also quantitative conclusions about the role of these antigens in the differentional diagnosis of Q fever .

The second protein group was tested by ELISA using only 1 negative blood donor sera (from Table 3) and 2 sera samples from chronic and acute Q fever patients, respectively (from Table 3). Lastly, IcmG and IcmK were tested only with 1 negative blood donor sera (from Table 3) and 1 sera sample from chronic and acute Q fever patients, respectively (from Table 3).

Initially, indirect ELISA technique was optimized by using CBU_1910. Different protein concentrations were used as well as different serum dilutions. According to those experiments, the titration of CBU_1910 and Q fever positive serum determined by indirect ELISA showed high levels of reactivity in nearly all different concentrations tested. In view of this result, the ideal protein antigen to be applied in the indirect ELISA with the highest reactivity along with the minimum “background” interference was defined at a protein concentration of 1 μg/L and a serum dilution of 1/100. We then tested each of the other proteins in separate and ended up at the same results.

3.8.1 ELISA results about the first protein group

Table 4 demonstrates a representative example about ELISA results of the first protein group. As it was mentioned in Pa. 3.5, 160 different sample sera were tested. A concentrated table of all results can be found in the supplementary materials (Table S5).

Table 4: Representative ELISA results about the first protein group.

		IFA Titre						
	Patient ID	**Phase I**	**Phase II**	**CBU_1910**	**CBU_1718**	**CBU_0092**	**CBU_0612**	**CBU_0937**
Healthy Blood Donor	2768	0	0	0.710	0.330	0.230	0.280	0.450
Other infection	54197	0	0	0.760	0.720	0.230	0.290	0.310
Rheumatoid Disease	SLE1W	0	0	0.692	0.631	0.448	0,82	0,403
Acute Q fever	2296	512	4096	0.710	0.300	0.200	0.350	0.180
Chronic Q fever	2299	4096	4096	3.400	3.190	0.410	1.410	0.300

As seen in this table, CBU_1910 responds to sera from healthy blood donor and patients suffering from other infection, rheumatoid disease and acute Q fever in a

similar way, while its response to serum from patient with the acute form of Q fever is much more intense. This finding indicates a high specifity of CBU_1910 to chronic Q fever. Similarly, CBU_1718 and CBU_0612 seem to react better with the chronic form of the disease, while CBU_0092 and CBU_0937 present alike reaction to serum from people suffering from rheumadoit disease and those suffering from chronic Q fever. Statistical analysis of all ELISA results can lead us to safe conclution.

3.8.2 ELISA results about the second protein group

Table 5 presents ELISA absorbances at 450 nm for each one of CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545, CBU_1398 and DotC. Sera from one healthy blood donor was used as negative and sera from two patients suffering from chronic and acute Q fever were used, respectively. The same negative serum was applied two times in the ELISA plate.

Table 5: ELISA results at 450nm for CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545, CBU_1398 and DotC.

		IFA Titre									
	Patient ID	**Phase I**	**Phase II**		**CBU_0271**	**CBU_0952**	**CBU_0891**	**CBU_1143**	**CBU_0545**	**CBU_1398**	**DotC**
Healthy Blood Donor	2766	0	0		0.583	0.357	0,115	0.114	0,434	0,317	0.224
					0.544	0.386	0,107	0,150	0,420	0,302	0.230
				AVG	0.564	0.371	0.111	0.132	0.427	0.309	0.227
Patients with Acute Q fever	2238	1024	2048		1.965	1.430	0.723	0.451	2.042	0.997	2.005
	2266	256	1024		2.087	0,851	0.554	0.346	2.573	2.763	1.960
				AVG	2.026	1.140	0.638	0.398	2.307	1.880	1.983
Patients with chronic Q fever	2052	4096	8192		2.905	1.387	1.511	1.351	3.042	3.600	3.330
	2316	32768	65536		2.946	2.470	0.916	2.739	3.402	3.083	3.314
				AVG	2.925	1.928	1.213	2.045	3.222	3.341	3.322

According to table 5, all proteins seem to be sensitive in both the acute and the chronic form of the disease, while they don't react with the serum of the healthy blood donor.

3.8.3 ELISA results about the third protein group

The third protein group tested by ELISA includes IcmG and IcmK, two proteins of *Coxiella*'s T4BSS. In this case, only one serum was used for each assay (negative, acute and chronic). Table 6 summaries the information about these sample sera and it also presents ELISA absorbance at 450 nm for each protein.

Table 6: ELISA results about IcmG and IcmK. Absorbance is measured at 450nm.

		IFA Titre			
	Patient ID	**Phase I**	**Phase II**	**IcmG**	**IcmK**
Healthy Blood Donor	2766	0	0	0.306	0.487
Patients with Acute Q fever	2238	1024	2048	0.810	0.459
Patients with Chronic Q fever	2316	32768	65536	1.688	1.760

Table 6 indicates that IcmG and IcmK respond to the chronic Q fever (with absorbances of 1.688 and 1.760 respectively) while IcmG seems to react with the serum of the patient suffering from acute Q fever (absorbance: 0,810).

3.9 Statistical Analysis

As it was mentioned above, statistical analysis was used only in the case of CBU_1910, CBU_1718, CBU_0092, CBU_0937 and CBU_0612. This procedure allowed us to calculate sensitivity, specificity, positive predictive value, negative predictive value and Cohen's kappa coefficient (κ) for each antigen. In addition, we performed binary logistic regression analysis to find any possible combinations of proteins that could act as better diagnostic factors.

To start with, the patients tested were initially divided into five groups:

a) chronic patients (based both on laboratory and clinical examination)

b) blood donors

c) patients whose samples were negative for phase I IgG

d) patients whose at least one sample was positive for phase I IgG and

e) patients suffering from any kind of rheumatoid diseases.

During the second step of analysis samples were divided into two categories, those presenting an IgG phase I titer of ≥ 1/1024 and those below this cut-off point.

The cut-off point for each protein was calculated based on this laboratory cut-off and on the distribution of the samples based on which corresponded to true positive chronic patients (Table 7 and Figure 25).

Table 7: The cut-offs calculated for each protein following the interpretation of the ELISA values against the IFA titres.

Protein properties and features	**ELISA results**
Gene ID/Protein	**Cut-off**
CBU_1910	1.88
CBU_1718	1.5895
CBU_0092	1.755
CBU_0937	1.2465
CBU_0612	0.62

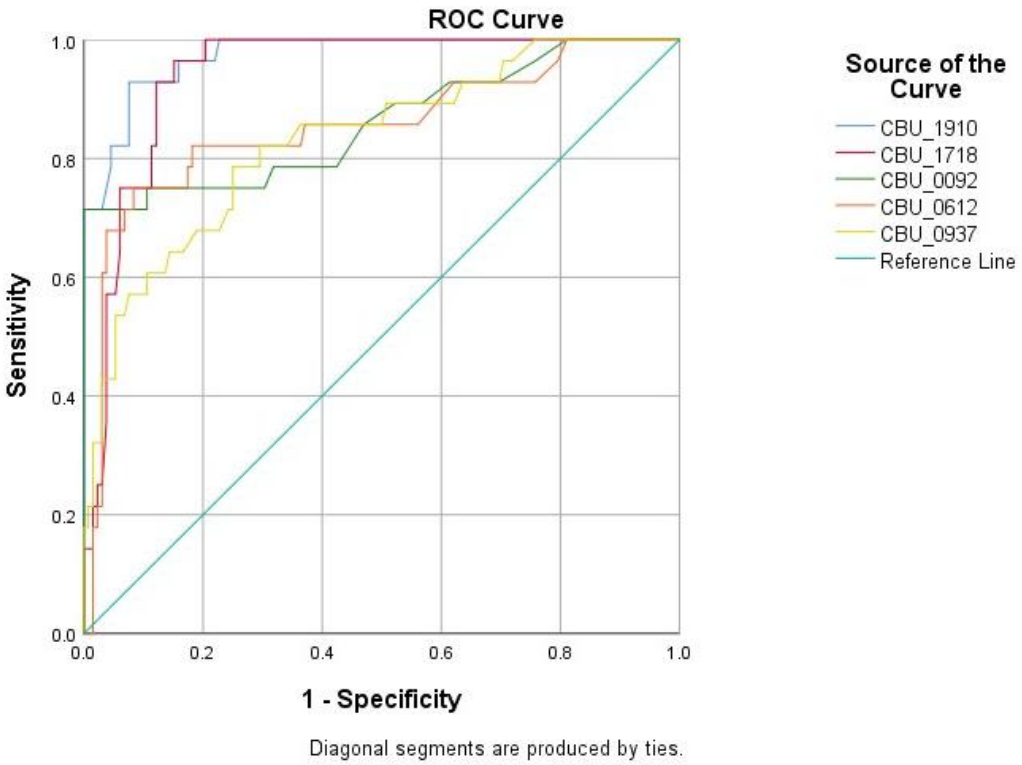

Figure 25: Specificity and sensitivity of each protein based on the ROC analysis.

Based on the proteins' cut-off, the discrepancy between the result obtained was calculated following the final diagnosis set up by the clinicians against that of the ELISA testing (Table 8 and Figure 25). From this Table it can be extracted that, for example in the case of the protein CBU_1910, 92.4% (122/132) of the samples tested agreed with the negative clinical diagnosis and 72.2% (26/36) of the samples tested agreed with the positive clinical diagnosis. Similar conclusions can be extracted for the rest of the proteins. Only protein CBU_0092 presents with a 100% agreement with clinical diagnosis demonstrating its high specificity.

Table 8: True and False Negative, True and False Positive values as calculated based on the ROC curve analysis for each protein in separate. The number of samples agreeing with the clinical diagnosis and presenting with proteins above or below the cut-off set up for each one in the current study can be extracted.

Protein	**Clinical diagnosis (Chronic Q fever)**	**ELISA**		
		Negative	Positive	Total
1910	Negative	122	10	132 (82.5%)
	Positive	2	26	28 (17.5%)
	Total	124 (77.5%)	36 (22.5%)	160
1718	Negative	112	20	132 (82.5%)
	Positive	1	27	28 (17.5%)
	Total	113 (70.6%)	47 (29.4%)	160
0092	Negative	132	0	132 (82.5%)
	Positive	8	20	28 (17.5%)
	Total	140 (87.5%)	20 (12.5%)	160
0612	Negative	121	11	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	128 (80%)	32 (20%)	160
0937	Negative	99	33	132 (82.5%)
	Positive	6	22	28 (17.5%)
	Total	105 (65.6%)	55 (34.6%)	160

Following the ROC curve analysis (Table 9), the true and false negative and the true and false positive values were calculated for each protein (Table S2).

Table 9: Values calculated for each protein based on the Area Under the Curve analysis. Significance was set at > 0.05. The test performance indicators calculated for each protein based on the comparison between the ELISA and the IFA testing are also shown. PPV: Positive Predictive Value. NPV: Negative Predictive Value.

Area under the curve										
				Asymptotic 95% confidence interval						
Proteins	Area	Std. Error	Asymptotic Sig.	Lower bound	Upper bound	**Sensitivity**	**Specificity**	**PPV (%)**	**NPV (%)**	**Kappa value (κ)**
CBU_1910	0,974	0,012	0,000	0,951	0,997	0,929	0,924	92,85	92,42	0,767
CBU_1718	0,942	0,018	0,000	0,907	0,976	0,964	0,848	96,42	84,84	0,641
CBU_0092	0,860	0,048	0,000	0,767	0,954	0,714	1,000	71,42	100	0,805
CBU_0612	0,852	0,047	0,000	0,760	0,944	0,750	0,917	75	91,66	0,631
CBU_0937	0,830	0,044	0,000	0,743	0,917	0,786	0,750	78,57	75	0,388

Furthermore, for all proteins, we have calculated a significant correlation to the presence of the disease (all p values were 0.00) while the area under the curve ranged from 0.83 (CBU_0937) to 0.942 (CBU_1718). Several other factors were, also, calculated including true rates, predictive values, likelihood ratios, the probability of agreement by chance and the κ coefficient (Table S2).

Apart from the ROC curve analysis we also tried to process the proteins following the binary regression analysis in order to search for possible combinations of the proteins that could increase their diagnostic value. For this purpose, all the above factors were again calculated (Tables S3, S4) for each individual protein (CBU_1910, CBU_1718, CBU_0092, CBU_0937, CBU_0612) and for any possible protein combinations (data in the supplementary materials)

For example, when we recalculate the results of protein CBU_1910 it can be seen that using the binary regression analysis 97% (128/132) instead of 92.4% of the samples tested agreed with the negative clinical diagnosis and 83.3% (20/24) instead of 72.2% of the samples tested agreed with the positive clinical diagnosis. However, it seems that whatever combination is used, very little changes are achieved in terms of true and false positive rates, compared to the values obtained for each protein alone (Table S4). Three more factors (B, exponential B and constant) were further calculated in an attempt to build up a model (Table S4). This attempt was not successful since whatever combination we tried the model kept working with one of the proteins used keeping the rest one(s) constant. As it seems, proteins CBU_1910, CBU_1718 and to a lesser extend CBU_0092 play the most important role in the correct diagnosis of the disease. In our case, the Exp(B) demonstrates the possibility for a person to suffer from the disease if his sample is detected over the cut-off for each protein compared to a person whose sample is tested below the cut-off set in the present for the same protein.

4. DISCUSSION AND CONCLUSION

Concerning the timely diagnosis of Q fever, a diagnostic problem exists since the currently gold standard method for the diagnosis, immunofluorescence, has several disadvantages. To mention just a few, the requirement of acute and convalescent sera, the objectivity of the interpretation of the results, potential antibody cross-reactions, the need for experienced personnel, etc. The main goal of this study is the development of a new, reliable and fast diagnostic tool against Q fever, which will be based on antibody detection. Even though there are commercially available diagnostic kits for several pathogens, such as Legionella [Bram et.al., 2006], Malaria parasite [Katakai et al., 2011] and Campylobacter [Day et al., 2012], there is no such a kit for the differential diagnosis of Q fever in human sera. Continuing previous studies done in our laboratory, we tested several proteins reported as antigens for their ability to differential diagnose the chronic or the acute form of the disease.

More specifically, CBU_1910, CBU_1718, CBU_0092, CBU_0612, CBU_0236 and CBU_0937 were chosen as potential chronic Q fever diagnostic markers, according to literature [Skultety et al, 2017, Xiong et al, 2012, Xile et al, 2013, Sekeyová et al, 2010, Kowalczewska et al, 2011, Papadioti, 2010]. Five of them (CBU_1910, CBU_1718, CBU_0092, CBU_0612 and CBU_0937) were successfully expressed and used for further studies. In a similar way, CBU_0271, CBU_0632, CBU_0952, CBU_0311, CBU_0229, CBU_0891, CBU_1143, CBU_0545 and CBU_1398 were chosen as acute Q fever potential biomarkers [Kowalczewska et al, 2011,Xiaolu Xiong et al, 2012]. Out of them, six targets (CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545 and CBU_1398) were successfully expressed and analyzed further.

In addition, three proteins from T4BSS of *Coxiella burnetii* (IcmG, IcmK and DotD) were analyzed for the same reason. Their antigenic properties haven't been described yet, however, the fact that T4BSS plays a crucial role in pathogen's virulence is well known [Voth et al, 2012, Carey et al, 2011]. This is why we decided to study these proteins for their ability to react with acute or chronic (or both) Q fever patients' antibodies.

In parallel, IcmG and IcmK were studied further on their biophysical properties, such as their oligomeric state, secondary structure and melting point. These particular

experiments lay the basis for structural characterisation and functional characterisation of IcmG and IcmK.

4.1 Exploring the antigenic properties of CBU_1910, CBU_1718, CBU_0092, CBU_0612 and CBU_0937

The group of these five proteins was chosen to be tested as potential chronic Q fever diagnostic markers. As it was mentioned in Results Section, we were able to express these targets in *E. coli* host cells, successfully purify them in one purification step (Ni-NTA affinity chromatography) and then proceed the study to western blot, ELISA assay and finally statistical analysis. According to the statistical analysis, all these proteins appear high sensitivity and specificity in the diagnosis of chronic Q fever. Among them, CBU_1910 (Com1),CBU_1718 (HspB) and CBU_0092 (YbgF) could be considered as more reliable antigenic candidates for the development of a chronic Q fever specific diagnostic tool.

CBU_1910 (Com1) is known to be preferentially exposed on the surface of *C. burnetii* [Hedrix et al, 1993] and is generally considered as a credible Q fever sero-diagnostic marker. According to Xiong et al, CBU_1718 (HspB) and CBU_0092 (YbgF) were recognized by both the blood sera of infected mice and humans. In addition, those two proteins have been previously described as major seroreactive antigens [Chao et al, 2005; Coleman et al, 2007, Sekeyova et al, 2009]. On the other hand, CBU_0612 (OmpH) is a 18 kDa outer membrane chaperon protein that is required for the release of the translocated proteins from the plasma membrane [Dumets et al, 2006]. Moreover, CBU_0612 (OmpH) has been documented as a very strong immunodominant marker for both acute and chronic form of the disease, according to Kowalczewska et al in 2012. CBU_0937 (UPF0422) was first described as an antigenic target for the diagnoses of Q fever by Sakeyova et al in 2010. According to their study, this protein appears to have low value of sensitivity but a high range of specificity against Q fever, which could lead to false negative results. However, the authors suggest that CBU_0937 (UPF0422) could be used together with Com1 as a usable tool in comparison with the commercial Q fever diagnose kit.

In addition, Skultety et al describe in their 2017 review that CBU_1718 (HspB) appears to be a very reliable molecular marker for serodiagnosis of both acute and chronic Q fever and CBU_1910 (Com1) acts as an antigen which might induce

protective immunity. Furthermore, it is suggested that CBU_0092 (YbgF) is a phase II specific marker that can be employed for early diagnosis of acute infection. According to the same review, CBU_0612 (OmpH) is a promising candidate marker for acute and chronic Q fever.

In this study, ELISA assay was used in order to screen blood sera from patients suffering from the chronic and the acute form of Q fever respectively. In general, the results concerning those experiments produced positive perspectives in Q fever differential diagnosis. In more detail, firstly the immonoblotting results (Figure 22) and secondly the ELISA assay (Table 4) indicate the qualitative conclusion that all these five targets appear to be sensitive in the differential diagnosis of chronic Q fever. In addition, statistical analysis confirmed those first outcomes by giving us quantitative results.

In the case of CBU_1910 (Com1), statistical data suggest that this protein is indeed immunologically responsive to sera from patients with chronic Q fever, while showing no response against sera from patients suffering from the acute form of the disease. The subsequent statistical analysis has shown that CBU_1910 (Com1) has sufficient specificity (92.4%) and sensitivity (92.9%) against the chronic form of the disease so that it can be considered as an antigen for the development of a chronic Q fever specific diagnostic tool. In the study of Beare et al of 2008, CBU_1910 (Com1) exhibits lower sensitivity and specificity against Q fever. This difference between those results and ours, is probably due to the fact that our research is focused on the chronic form of Q fever and thus studying the ability of the protein to adequately diagnose the particular form of the disease. Proceeding further, referring to the four other proteins (CBU_1718, CBU_0092, CBU_0612 and CBU_0937), the results shown above confirm that all of these antigens respond to sera from patients suffering from the chronic form of the disease, while they appear to have been limited to no response to sera from patients with acute Q fever. The statistical analysis suggested high sensitivity and specificity to chronic Q fever for CBU_1718 (HspB) (Table 9), while CBU_0092 (YbgF) and CBU_0612 (OmpH) appear to be specific but not sensitive enough (Table 9), a fact that may lead to false negative results.

As a conclusion, CBU_1910 (Com1), CBU_1718 (HspB) and CBU_0092 (YbgF) could be considered as reliable antigenic candidates for the development of a chronic Q fever specific diagnostic tool.

The outcome of this part of the study could be really beneficial not only for scientific knowledge but also for public health since fast and reliable diagnosis, as well as the valid differentiation between the acute and the chronic type of the disease, play a key role in the progress of Q fever disease. Improving the gold standard method for the diagnosis by using the current results of the study would help health professionals choose the correct antibiotic treatment, based on the type of Q fever (acute or chronic). The difference is fundamental, as the chronic Q fever treatment protocol requires systematic re-examinations and treatment lasting at least 18 months. On the other hand, there are many cases of patients being mistreated because of the false diagnosis of the form of the disease. Furthermore, late diagnosis of Q fever could be life threatening.

4.2 Exploring the antigenic properties of CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545, CBU_1398 and DotC

CBU_0271 (ssb), CBU_0952, CBU_0891, CBU_1143 (yazC), CBU_0545 (LemA) and CBU_1398 (sucB) are six proteins that have been described to be acute Q fever specific. In more detail, CBU_0271 (ssb) is as being the second best diagnostic biomarker for the case of acute Q fever, after CBU_0092 (YbgF) according to Kowalczewska et al. In addition, Raoult and co-workers in their research of 2011 outline CBU_0952 as an acute disease antigen and also as a serodiagnostic subunit for acute Q fever diagnosis. On the other hand, the same protein has also been suggested a promising marker for patients with Q fever endocarditis.

In the same study, CBU_0891, CBU_1143 (yazC) andCBU_0545 (LemA) have been described as three of the best markers for Q fever in general, while CBU_1143 (yazC) has been suggested in the Skultety's review (2017) not only as the most frequently reactive antigen, but also a potential candidates for subunit vaccines. Furthermore, CBU_1398 (sucB) is described to be phase II specific and also a marker for the diagnosis of early stage acute Q fever. This target has also been suggested as a good antigen for serodiagnosis or vaccine development [Raoult et al, 2011].

Moreover, DotC is a 30 kDa outer-membrane protein which belongs to Dot/ Icm T4SS of *Coxiella burnetii*. According to literature, DotC, along with DotD and DotH (IcmK), is essential for the formation of the Dot/Icm core complex and form an ring-shaped outer-membrane subcomplex [Kubori et al, 2014]. Its antigenic abilities haven't been described before.

In this particular study, CBU_0271 (ssb), CBU_0952, CBU_0891, CBU_1143 (yazC), CBU_0545 (LemA), CBU_1398 (sucB) and DotC have been expressed in *E. coli* host cells, isolated and purified in one step affinity chromatography. However, we weren't able to manage a great yield and a high purity, apart from the case of DotC that SDS-PAGE gel shows its quite good purity after affinity chromatography (Figure 14). After isolation, these targets were analyzed by western blot and ELISA, resulting in qualitative conclusions about their ability to differential diagnose Q fever disease.

One negative serum and two different sera from patients with chronic and acute Q fever respectively, were used for the western blot and ELISA assay in order to test the antigenicity of the proteins. More specifically, in the case of immunoblotting, a mixture of the two sera from patients with the acute form of the disease and a mixture of the two sera from patients suffering from chronic Q fever were used against the targets. On the other hand, different sera were used separately in the ELISA assay.

Taking into consideration the data on Figure 23 and Table 5, we can conclude that western blot and ELISA results seem to agree in general. In more detail, CBU_0271 (ssb), CBU_0952 , CBU_0891, CBU_1143 (yazC) and CBU_0545 (LemA) appear to react with sera from both acute and chronic Q fever, with the signal of CBU_0891 and CBU_1143 (yazC) being poor with the acute Q fever sera in the western blot. Indeed, Table 5 indicates that all proteins, apart from CBU_0891 and CBU_1143 (yazC), show high absorbance for sera from patients suffering from both the acute and the chronic form of the disease (in comparison with the negative serum). Also, in Figure 23, CBU_1398 (sucB) seems to have no reaction with acute Q fever, while in ELISA results the average absorbance of the target with sera from patients with the acute form of the disease is relatively high (1.880). This might be due to the fact that in western blot, antibodies interact with the unfolded protein (primary structure) while in ELISA the purified recombinant protein (antigen) is completely folded (secondary

structure). As a result, antibodies in serum from patient with chronic Q fever might have reacted more effectively with the fully folded antigen (ELISA) rather than the unfolded one in western blot.

On the other hand, western blot of Figure 23 demonstrates that chronic Q fever sera have reacted not only with the target proteins, but also with the impurities of protein samples, while acute Q fever sera react only with our targets. This is a point to conclude that impurities in protein samples affect ELISA results by increasing the absorbencies for the case of chronic Q fever, while high absorbance for acute Q fever sera are exclusively due to the reaction of the targets with the sera from patients suffering from the acute form of the disease. However, these are the primary results about the study of the antigens specific in the acute form of Q fever, so more experiments must take place in order for these proteins to be isolated in a really high yield and purity and be analysed further for their ability to differential diagnose acute Q fever.

Figure 24 demonstrates the immunoblotting of DotC reacting with sera from patients suffering from acute and chronic Q fever respectively. As it shows, DotC appears a very faint signal in the case of chronic Q fever sera, while it demonstrates no reaction with acute Q fever sera. This result does not agree with the ELISA assay. In Table 5, we can see high average absorbancies in sera from patients suffering from both chronic and acute Q fever (3.322 and 1.983 respectively while 0.227 is the absorbance for the negative serum). Again in this case, this discrepancy might be caused by the different interaction ability between DotC and serum antibodies when the antigen is completely folded (ELISA) and unfolded (Western blot), as described above.

4.3 Exploring the antigenic properties of IcmG and IcmK

After the expression and purification of IcmG and IcmK in large amounts and high purity, western blot and ELISA assay took place in order to study their ability to diagnose Q fever. Figure 24 and Table 6 demonstrate those results. At this point, we should mention that sera used in the western blot and ELISA were from different patients, contrary to the case of the other targets, where sera from the same patients suffering from acute or chronic Q fever were tested in both cases.

According to immunoblotting (Figure 24), both proteins seem to react with sera from patients suffering from the chronic or the acute form of the disease. Table 6 indicates that indeed, IcmG and IcmK appear to have some antigenic properties. In more detail, ELISA results (Table 6) demonstrate that IcmK seems to be specific to the chronic form of the disease, as the absorbance in this case is 1.760 while in the case of serum from the acute Q fever the absorbance is 0.459, almost the same as the negative sample (healthy blood donor).

On the other hand, IcmG shows antigenicity to both acute and chronic Q fever, with a slight preference to the chronic form of the disease. Absorbance of serum from the patient suffering from chronic Q fever is 1.688 while the corresponding absorbance to serum from acute Q fever patient is 0.810 and the negative sample gives a value of 0.306. These results suggest for the very first time that proteins of the sectretion system can act as antigens.

In any case, further investigation is needed in order to come to safer conclusions about the selectivity of IcmG and IcmK for the different disease forms.

Consiquently, our data about the second and the third protein group that was studied, indicate that these proteins could serve as promising candidates for the diagnosis of acute Q fever, while IcmG, IcmK and DotC, proteins from the Dot/Icm secretion system of Coxiella burnetii appear antigenicity against sera from patients suffering from chronic or acute Q fever.

4.4 Clinical results

During this sudy, 14 proteins were chosen and tested for their ability to differential diagnose Q fever disease in patients' sample sera. In addition, 160 sample sera were used, including patients' suffering from acute Q fever, patients suffering from the chronic form of the disease, healthy blood donors and patients suffering from rheumatoid disease. Table S5 (supplementary material) presents all these sample sera categories, identifying their laboratory diagnosis titers for PhI and PhII IgG as well as ELISA absorbencies when the proteins of interests act as antigens. According to this table, the higher the titer is, the more "antigenic" the protein appears to be, demonstrating higher ELISA absorbencies. Moreover, according to the statistical

analysis that took place for 5 out of 14 proteins, the diagnostic ability of these antigens agrees with the laboratory diagnosis, based on IFA. In fact, these 5 proteins demonstrate high sensitivity and specificity (> 50%) against the chronic Q fever, a fact that makes them great candidates for the development of a new, reliable, ELISA based diagnostic tool.

4.5 Oligomer state of IcmG and IcmK

BN-PAGE was performed in order to investigate the oligomer state of IcmG and IcmK. The oligomer state of IcmG in four different detergents was analyzed, as it has been reported that detergents can influence the oligomer state of membrane proteins [Reading et al, 2016].

According to BN-PAGE gel (Figure 15), IcmK is present mainly in monomers. On the other hand, in the case of IcmG purified with LDAO, several protein bands with different molecular masses can be observed (Figure 15), suggesting the presence of monomers and dimers of the IcmG. Furthermore, additional bands at higher molecular weights may also indicate the formation of high oligomeric complexes of IcmG. Further investigation was carried out in order to discover how different detergents affect oligomer state of a protein. To this purpose, LDAO detergent was exchanged with DDM, Fos-Cholin 12 and Fos-Cholin 14 by using a Strep-Tactin affinity chromatography.

As Figure 15 indicates, the pattern of IcmG oligomeric state seems quite similar in all these three detergents.The small differences are probably due to the different mass and number of the bound detergent to the protein.

4.6 Characterisation of IcmG and IcmK

IcmG and IcmK were analyzed by DSF, DLS and far- UV CD spectroscopy in order to determine some of their characteristics, such as their melting point (T_m), their hydrodynamic dimension and their secondary structure folding. Figures 16-21 illustrate the results of these studies.

Initially, the particle size of IcmK was determined to be 3.9±0.06 nm, suggesting that this protein may exist in a monomeric form (Figure 16), which agrees with the results observed from the BN-PAGE analysis. In addition, the determination of the

secondary structure (Pa 3.5.2) of IcmK, according to the CD spectrum (Figure 18) correlates with the finding after BLAST alignment that some regions of the protein may have disordered structure.

DSF is a method which is used as a screening tool that helps researchers to find conditions where a protein of interest is optimally stable and therefore more likely to crystallize. Additionally, the method helps scientists to evaluate purification and storage conditions. According to Figure 20, IcmK exhibits a pretty high melting point (T_m) of about 56°C, which means that the protein remains stable at both 4^{o}C and room temperature, making its handling easier.

Concerning IcmG, which is a membrane protein, all tests mentioned above were performed in the presence of the four different detergents (LDAO, DDM, Fos-Cholin 12, Fos-Cholin 14). DLS results (Pa 3.5.2- Figure 17) are reasonable, as the variation of the size correlates with the difference size of the detergents micelle [Chaptal et al, 2017]. More specifically, the bigger the size of the detergent micelle is, the larger the particle size of the membrane protein is. This is the case with IcmG in those four different detergents, as DDM formats the largest micelle and LDAO the smallest [Stetsenko and Guskov, 2017, Chaptal et al, 2017].

Moreover, CD spectroscopy was applied in order to characterize the secondary structure of IcmG (Figure 19). The CD data (Pa 3.5.2) show that a high percentage of flexible structure is present in the isolated protein, however it should be mentioned that this could be changed, since the proteins interact with other subunits of the T4BSS to form the intact secretion system (Pa. 1.5.1 and 1.5.2).

In Figure 21 we can see the melting point value of the IcmG protein in DDM. A study in 2011 [Dupuex et al, 2011] established a correlation between complex multiphasic denaturation behavior and a low likelihood of success in protein crystallization. Further studies have shown that proteins with a T_m higher than 45°C have a higher success rate in crystallization than proteins with lower melting temperatures [Dupuex et al, 2011]. Since both proteins of our interest have T_m higher than 45°C, there is a good chance for successful crystallization of these proteins in the future.

5. CONCLUSION

Q fever is a worldwide zoonosis that is caused by the intracellular bacteria *Coxiella burnetii*. *Coxiella burnetii* has been identified in a wide range of wild and domestic animals, including arthropods, birds, rodents, carnivores, ungulates and livestock, while it can be transmitted to humans via aerosols or direct contact with infected animals' excretions. Q fever has various acute and chronic clinical manifestations, with acute Q fever being mostly a self-limiting, mild, influenza-like disease, sometimes complicated by severe pneumonia or hepatitis. Due to these heterogeneous symptoms diagnosis usually delays from 1-14 months. Chronic Q fever, on the other hand, leads to high death rates if left untreated, which makes early case finding and preventive measures critical for patients at high risk. Q fever has a high socioeconomic burden that presents significant challenges for both public and animal health.

Regarding the timely diagnosis of chronic Q fever a diagnostic problem exists since the currently gold standard method for the diagnosis, immunofluorescence, seems to show several disadvantages, such as the requirement of acute and convalescent sera, the objectivity of the interpretation of the results, potential antibody cross-reactions, the need for experienced personnel etc.

This study focuses on the investigation of several proteins and to their ability to differential diagnose Q fever in patients sample sera, while its ultimate goal is the development of a reliable diagnostic tool for the chronic or the acute form of Q fever.

In summary, three different protein groups were tested. The first one included antigens that have been mentioned as chronic Q fever specific in the literature (CBU_1910, CBU_1718, CBU_0092, CBU_0612 and CBU_0937), the second group was consisted of proteins with specificity in the acute form of the disease (CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545 and CBU_1398) while the third group included three proteins from the T4BSS of *Coxiella burnetii* (IcmG, IcmK and DotC). In parallel, these three proteins were further studied for their biophysical properties, in a trial to provide an essential basis for determining the structure of these proteins.

The findings of this study are very promising in respect to the main goal. CBU_1910, CBU_1718, CBU_0092, CBU_0612 and CBU_0937 were established as very good chronic Q fever disease biomarkers, providing a basis for the development of a reliable and easy-to-use diagnostic tool for the chronic form of the disease, based on antigenic protein detection.

Last but not least, the findings about IcmG and IcmK biophysical properties might provide a good basis in the deciphering of the structures of these proteins in high-resolution. Subsequently, this will shed light on the molecular mechanisms of these two components of the T4BSS and will aid towards the development of drug therapies against *Coxiella*.

The findings of this study lay the basis for the development of a new, sensitive, and specific tool for the differential diagnosis of Q fever. Using the results of this research, and particularly the great sensitivity and specificity of Com1, HspB and YbgF, a reliable, ELISA-based diagnostic kit could be manufactured. In this way, laboratory diagnosis could be easier, especially in laboratories that are not able to grow *C.burnetii* and use it as an antigen. Consequently, this work could be beneficial not only for the basic research, but also for public health, introducing new perspectives in the development of diagnostic tools against Coxiella.

FUTURE WORK

Future research should focus on the antigens that have a potential specificity in the acute form of Q fever. Studies could investigate further the exact role of CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545, CBU_1398 as well as IcmG, IcmK and DotC in Q fever diagnosis. In more detail, future research should include the following:

a) Optimization of expression and purification of CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545 and CBU_1398. High yields and purity of the antigens can lead us to more secure conclusions about their ability to diagnose acute or chronic Q fever.
b) New cloning experiments on the targets that we weren’t able to clone.

c) Testing more sample sera via the ELISA assay. After successful expression and purification of CBU_0271, CBU_0952, CBU_0891, CBU_1143, CBU_0545 and CBU_1398, a large number of sample sera should be tested with ELISA in order to collect enough data for statistical analysis. Testing sera could include sera from patients with acute or chronic Q fever as well as sera from healthy blood donors or patients suffering from other diseases. This step should be performed also for IcmG, IcmK and DotC, investigating their diagnostic abilities.

d) After collecting enough data from the ELISA assay, a statistical analysis should be performed. By this method, our targets' specificity and sensitivity in acute or chronic Q fever could be calculated and leading to interesting results.

e) In addition, more proteins for T4BSS should be tested for their antigenic propertie, such as the transmembrane protein IcmE or the lipoprotein DotD.

f) Further literature search is required, in order to find more proteins suggested as acute Q fever specific as well as proteins that have been reported as good biomarkers for early *Coxiella burnetii* infection (before symptoms appearance).

g) Solving the structure of IcmG and IcmK by high resolution by X-Ray Chrystallography is one of our major future goals.

h) The development of a rapid diagnostic tool for the differential diagnosis of Q fever.

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Supplementary Material

Primers Design

PCR primers were designed with a sequence overlap with both vector and insert of 15-30 bp respectively and they are listed in Table S1.

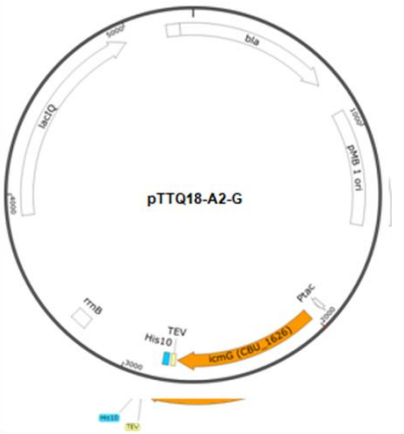
Table S1: PCR primers designed and used for the construction of the vectors

Primers	
PCR primer name	**Sequence (5'-3')**
CBU-1_for (IcmG)	CGGGATCCTGAGTATCGAAAATCAGAAAACCAT
CBU-2_rev (IcmG)	CGGAATTVGTACGATCATTAGCTCCATAAC
CBU-5_for (IcmK)	CGGGATCCTGATAAAGTGAGAAGATGGTG
CBU-6_rev (IcmK)	CGGAATTCGTCAACCCTTCAATCATCAACT
G-DelHis-F (IcmG)	AGTATCGAAAATCAGAAAACCATC
G-DelHis-R (IcmG)	CATCGCTGTTTCCTGTTGAAATTG
K-DelHis-F (IcmK)	AGTAAAGTGAGAAAAGATFFTTGATTC
K-DelHis-R (IcmK)	CATCGCTGTTTCCTGTGTGAAATTG
CD-strep_Fw (DotC)	CCCGCAGTTCGAAAAATAAGCTTGGCACTGGCCGTCGTTTTAC
C-strep_rev (DotC)	TGGCTCCAAGCGCTTGGCGAACTAGCCCCATCCGTCAAAACG
CBU_1718_Fw	GCCCATATGGCTGCAAAAGTTTTAAAATTTTCCCACGAGG

CBU_1718_Rev	TTACTCGAGCATCATGCCGCCCATGCCGCCCATTC
CBU_0092_Fw	GCCCATATGAGGCTTATTAAAATGAAAATAAAAACG
CBU_0092_Rev	TTACTCGAGAGGCGTTGTTGTTGCTGAATCGACTTC
CBU_0937_Fw	GCCCATATGACGTCCAAGCTGGTCATTTCCGCGTTGGGC
CBU_0937_Rev	TTACTCGEGAAAATAAAGATCGAACTGTGCCGTTACCAC
CBU_0612_Fw	GCCCATATGATTAAACGCTTGTTATCTGCTATCTG
CBU_0612_Rev	TTACTCGAGTTTCAATGCTGATACAACGTTTGAAG
CBU_0937_pelB_Fw	CCGGCGATGGCCATGAGTACCCCCGCTACCACTAATC
CBU_0937_pelB_Rev	GGTGGTGGTGCTCGAGAAAATAAAGATCGAACTGTGC
CBU_1718_Fw	GCCCATATGGCTGCAAAAGTTTTAAAATTTTCCCACGAGG
CBU_1718_Rev	TTACTCGAGCATCATGCCGCCCATGCCGCCCATTC
11_fw (CBU_0271)	GCCCATATGGGTGTAAATAAAGTTATTTTAATCG
11_rev (CBU_0271)	TATCCTCGAGAAACGGAATATCATCATCAAAATCGGCCAC
12_fw (CBU_0632)	GCCCATATGACTAGCGAAAATTACCATGAGCCAATAGATG
12_rev (CBU_0632)	TATCTCGAGTTCACCCGAGTGGTGATCCACAATAGGCTTATCTG

13_fw (CBU_0952)	GCCCATATGAAAAAACTAACCGTAACTTTCTTAACTTTTATTAG
13_rev (CBU_0952)	CTCGAGTTTATTTAAAAAAGTCGCCACTTTTACATTC
15_fw (CBU_0311)	GCCCATATGGAAACAACTACAAAACTCGCCATAGGCGTTTC
15_ rev (CBU_0311)	CTCGAGGATATTGAACAGGTAATTAACGGTTGCGGTAATG
16_fw (CBU_0229)	GCCCATATGGCACAATTATCAAAAGATGACATTTTAGAAGC
16_rev (CBU_0229)	CTCGAGTTCCAACTCGACTTTGGCACCTGCTTCTTC
17_fw (CBU_0891)	GCCCATATGAAACGGTATCTTTTTTTTACTATTATCTTTTTC
17_rev (CBU_0891)	CTCGAGAGGCAGGCTCTTTAAAAGCTCATCGGCCTCC
18_fw (CBU_1143)	GCCCATATGAGTCTATTGAGTGTATTAGGCGTAGGGGTCG
18_rev (CBU_1143)	CTCGAGAATCGAATCGATCGTGCCTTTGGGCAATAC
19_fw (CBU_0545)	GCCCATATGTCAAATCTTGCGTTTCCCACGATAGGCC
19_rev (CBU_0545)	CTCGAGCAAGTTTACTTTATAATCTTCTTGCTTAATTATTTTC
20_fw (CBU_1398)	GCCCATATGGCTATAGAAATTAAAGTACCTACGTTGCCTG
20_rev (CBU_1398)	CTCGAGACACTCCAAAATCATTCGAGCTGGATCTTCC

All plasmids constructed for this study are illustrated in Figure S1.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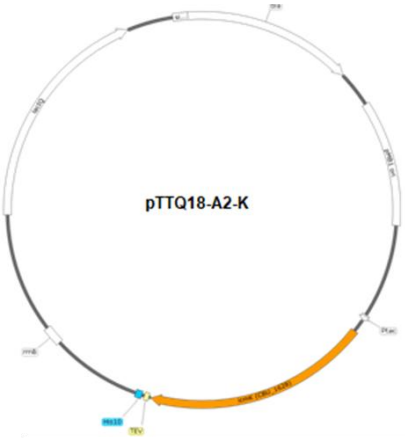
lacIQ
pMB 1 ori
rrnB
TEV
His10
icmG (CBU_1626)
Ptac

A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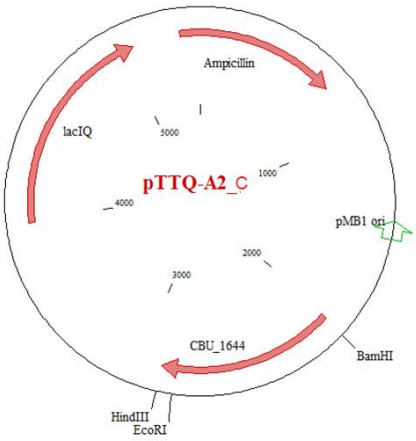
B


Ampicillin
lacIQ
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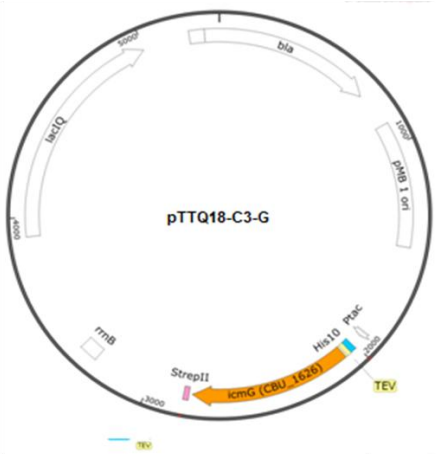
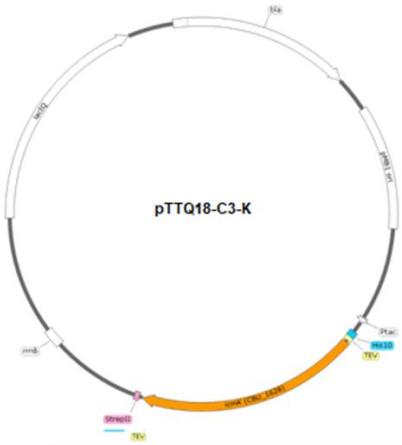
pTTQ-A2_C
1000
4000
pMB1 ori
2000
3000
CBU_1644
BamHI
HindIII
EcoRI

C


pTTQ18-C3-G
bla
lacIQ
pMB 1 ori
rrnB
StrepII
His10
Ptac
icmG (CBU_1626)
TEV

D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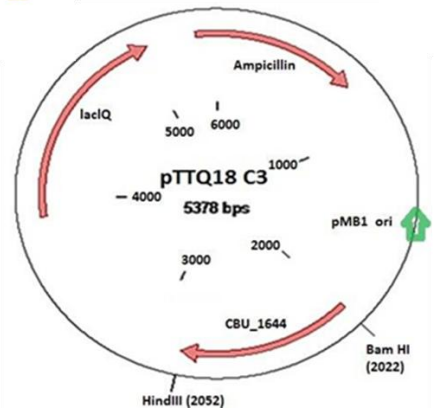
E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6000
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4000
5378 bps
pMB1 ori
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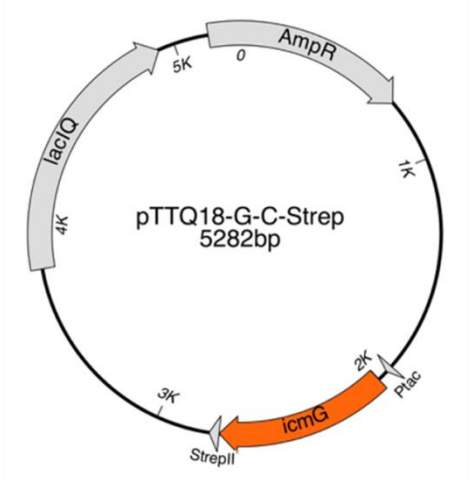
3000
CBU_1644
Bam HI
(2022)
HindIII (2052)

F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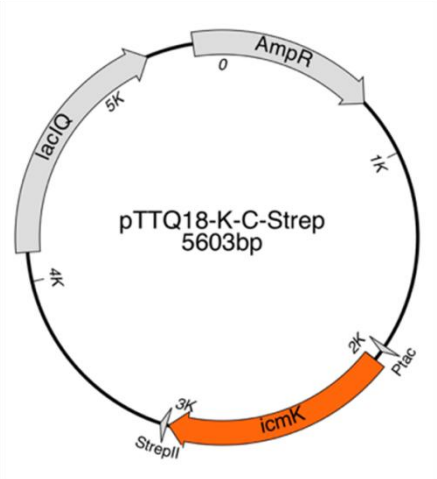
G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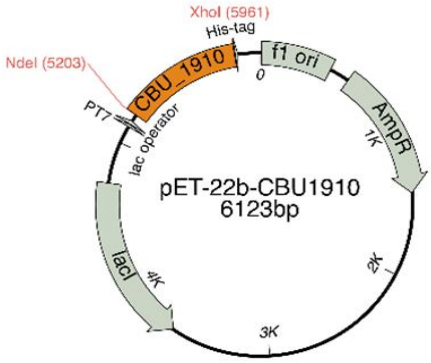
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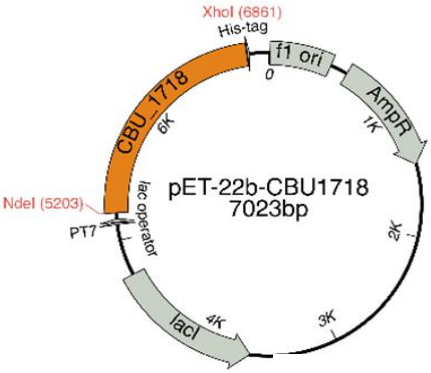
3K

I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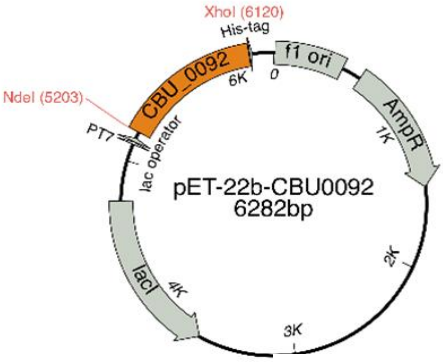
J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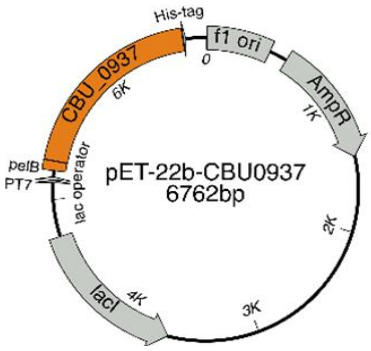
3K

K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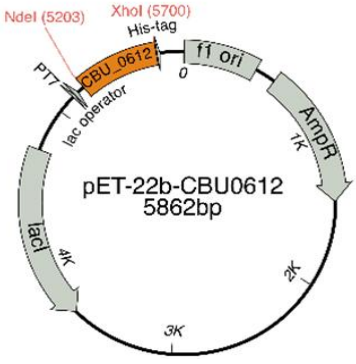
L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3K

M

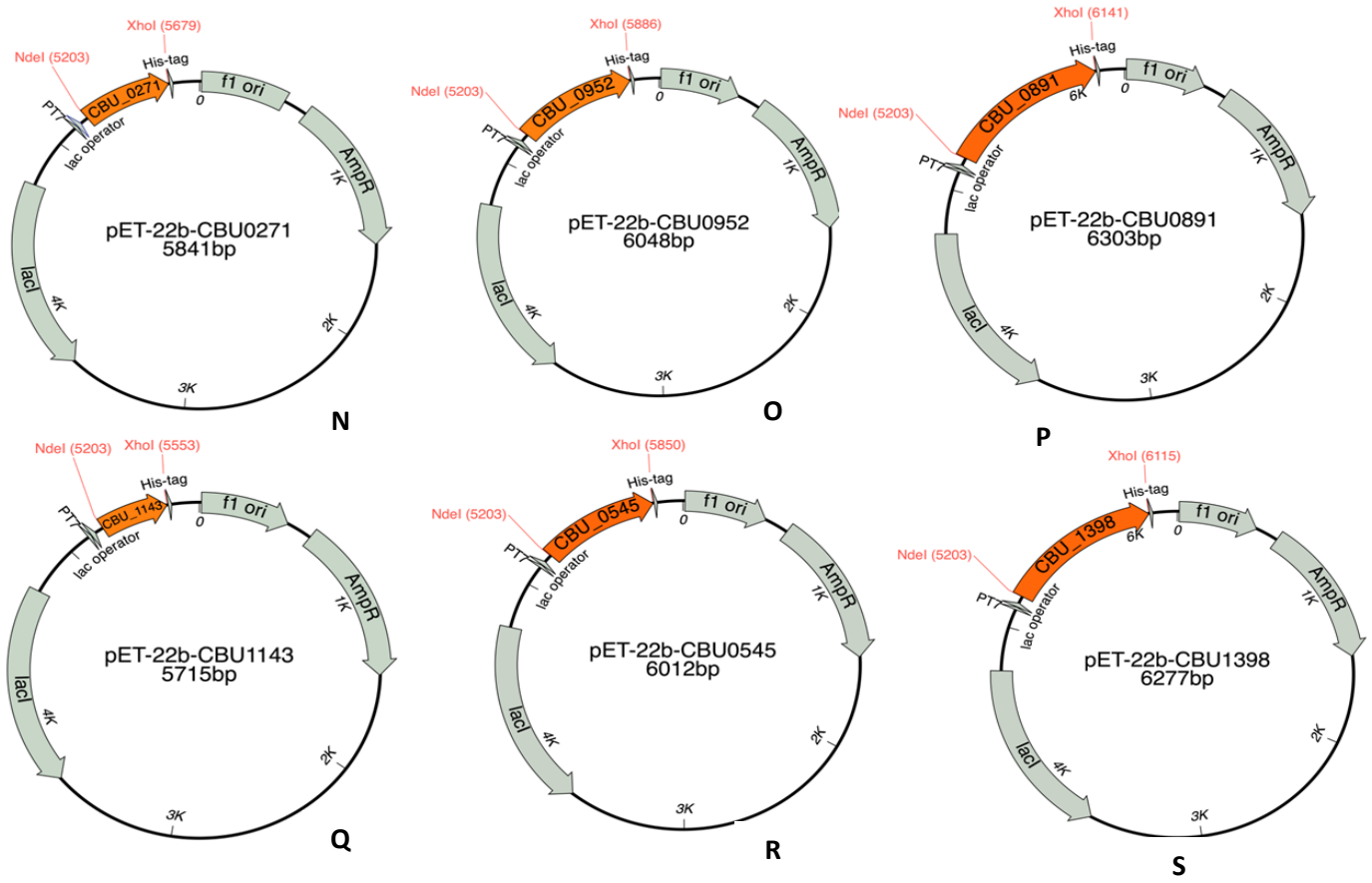

Figure S1: All vectors constructed in this study. **A**. pTTQ18A2- IcmG, **B**. pTTQ18A2-IcmK, **C.** pTTQ18A2- DotC**, D**. pTTQ18C3- IcmG, **E**. pTTQ18C3-IcmK, **F**. pTTQ18C3- DotC, **G.** pTTQ18 –G-C-strep, **H.** pTTQ18-K-C-Strep, **I**. pet-22b- CBU_1910, **J**. pet-22b-CBU_1718, **K**. pet-22b- CBU_0092, **L**. pet-22b- CBU_0937, **M**. pet-22b- CBU_0612, **N**. pet-22b-CBU_0271, **O**. pet-22b-CBU_0952, **P**. pet-22b-CBU_0891, **Q**. pet-22b-CBU_1143, **R**. pet-22b-CBU_0545, **S**. pet-22b-CBU_1396.

Figure S2 illustrates isolated IcmG after affinity chromatography. Membranes have been solubilized by five different detergents (1% (w/v) DDM, DDM/LDAO, LDAO, Octyl Glucoside and Triton.

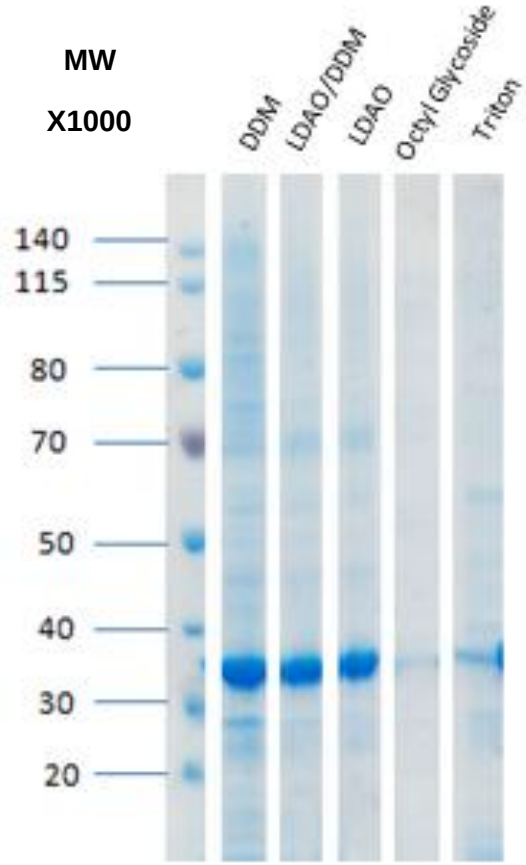

Figure S2: IcmG isolated by affinity chromatography. Membranes have been solubilized by five different detergents. LDAO was chosen as the best detergent for solubilization because SDS-PAGE result indicates isolated protein with the least contaminations in comparison with the four other detergents.

In Figure S3, we can see DSF results for pure IcmG in DDM, LDAO, Fos-Cholin 12 and Fos-Cholin 14. Although T_m for IcmG was observed in LDAO, only in DDM the protein has showed a sharp peak, indicating that in this detergent IcmG is more stable.

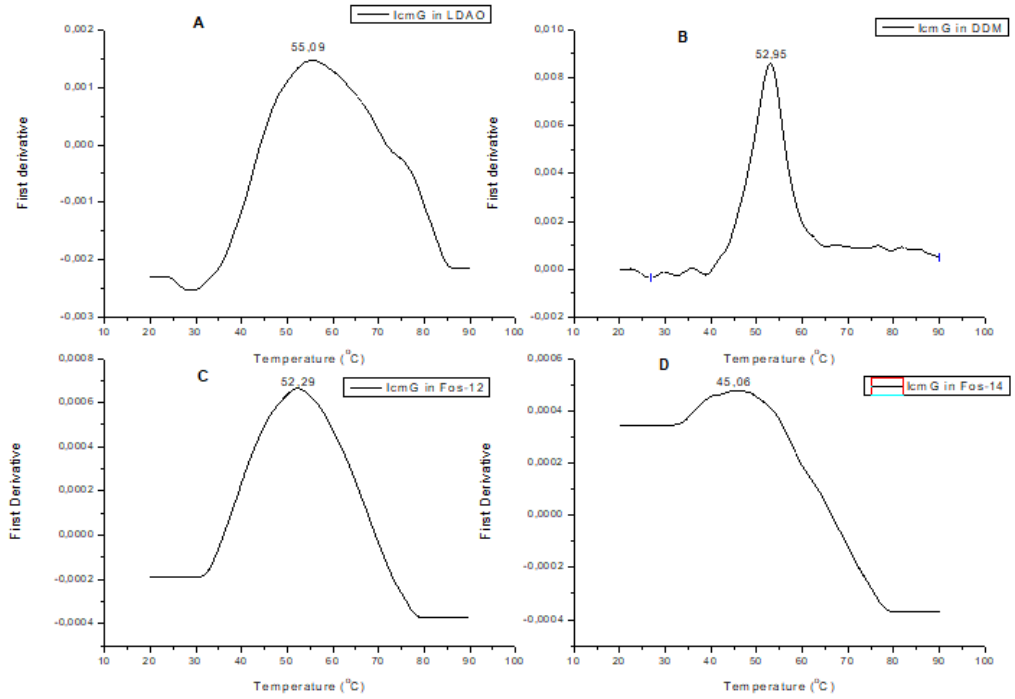

Figure S3: DSF results for IcmG in 4 different detergents, Pannel B is also shown in the results section (Figure 16.E).

Figure S4 shows SDS-PAGE and western blot results after the purification of the second group of proteins (Pa 3.8). The antibody used in western blot was anti-polyHistidine- Alkaline Phosphate antibody.

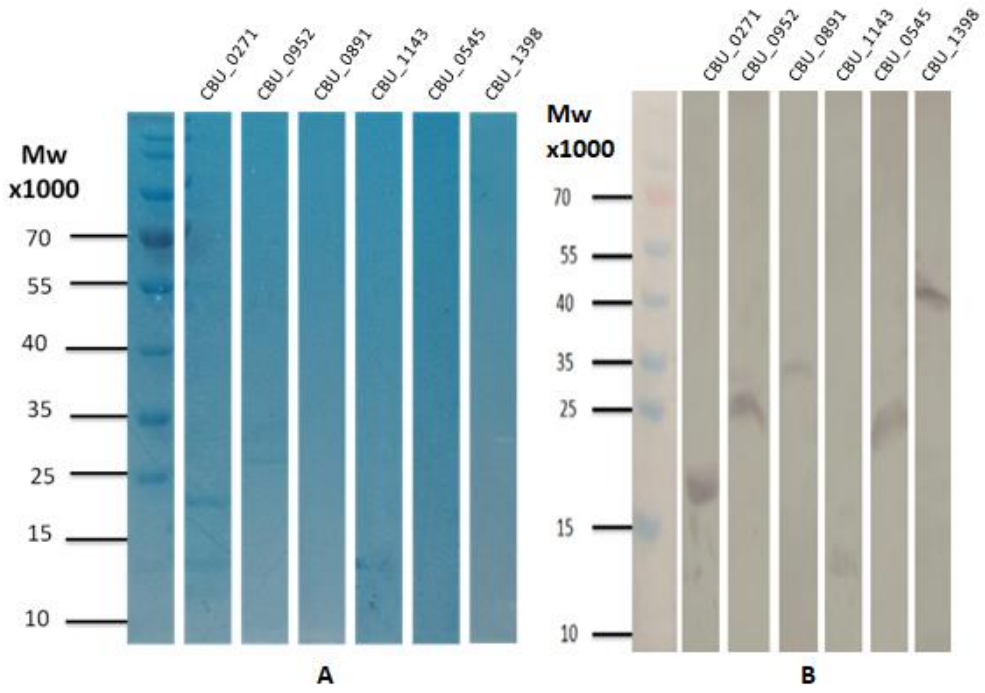

Figure S4: A. SDS-PAGE gel after affinity chromatography and **B.** Western blot using anti-polyHistidine- Alkaline Phosphate antibody.

Table S2: Statistical factors associated with the comparison between the indirect immunofluorescence antibody test (IFAT) and the recombinant protein-based ELISA for the detection of serum phase I IgG antibodies against C. burnetii. The statistical analysis was performed based on the ROC. TPR: True Positive Rate = sensitivity = probability of detection = power = tpr = tp/(tp+fn). TNR: True Negative Rate = specificity = selectivity = true negative rate = tnr = tn/(tn+fp). PPV: Positive Predictive Value = 1-specificity = precision = confidence = tp/(tp+fp). NPV: Negative Predictive Value = tn/(tn+fn). FPR: false positive rate = probability of false alarm, fpr= fp/(fp+tn). FNR: false negative rate = miss rate = fnr = fn/(fn+tp). lr+: positive likelihood ratio = lr+ = tpr/fpr. lr negative likelihood ratio = lr- = fnr/tnr. OR: Odds ratio = lr+/lr-. Acc: accuracy = classification rate, acc = (tp+tn)/(tp+tn+fp+fn). 95% CI= 95% Confidence Interval. PE: the probability of agreement by chance, pe = [(tp+fp)x(tp+fn)+(tn+fp)x(tn+fn)]/[(tp+tn+fp+fn)**2]. κ: Cohen's kappa coefficient of agreement, κ = (acc-pe)/(1-pe).

Protein	TPR	TNR	PPV	NPV	FPR	FNR	lr+	lr-	OR	Acc	95% CI	PE	K
1910	0,929	0,924	0,722	0,984	0,076	0,071	12,257	0,077	158,6	0,925	32.8-767	0,679	0,767
1718	0,964	0,848	0,574	0,991	0,152	0,036	6,364	0,042	151,2	0,869	19.4-1176.7	0,634	0,641
0092	0,714	1,000	1,000	0,943	0,000	0,286	-	0,286	-	0,950	-	0,744	0,805
0612	0,750	0,917	0,656	0,945	0,083	0,250	9	0,273	33	0,888	11.5-94.8	0,695	0,631
0937	0,786	0,750	0,400	0,943	0,250	0,214	3,143	0,286	11	0,756	4.1-29.5	0,602	0,388

Table S3: True and False Negative, True and False Positive values as calculated based on the Binary logistic regression analysis for each protein in separate and for the combinations of the proteins. No results have been calculated for the combination of the proteins 1718+0092, 1718+0092+0612 and the combination of all the proteins without 1910 because the Hosmer and Lemeshow test failed. From the Table the number of samples agreeing with the clinical diagnosis and presenting with proteins above or below the cut-off set up for each one in the current study can be extracted.

Protein or combinations	**Clinical diagnosis**	**ELISA**		
		Negative	Positive	Total
1910	Negative	128	4	132 (82.5%)
	Positive	8	20	28 (17.5%)
	Total	136 (85%)	24 (15%)	160
1718	Negative	124	8	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	131 (81.9%)	29 (18.1%)	160
0092	Negative	132	0	132 (82.5%)
	Positive	8	20	28 (17.5%)
	Total	140 (87.5%)	20 (12.5%)	160
0612	Negative	127	5	132 (82.5%)
	Positive	9	19	28 (17.5%)
	Total	136 (85%)	24 (15%)	160
0937	Negative	128	4	132 (82.5%)
	Positive	16	12	28 (17.5%)
	Total	144 (90%)	16 (10%)	160
1910+1718	Negative	129	3	132 (82.5%)
	Positive	6	22	28 (17.5%)
	Total	135 (84.4%)	25 (15.6%)	160
1910+0092	Negative	130	2	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	137 (85.6%)	23 (14.4%)	160
1910+0612	Negative	130	2	132 (82.5%)
	Positive	8	20	28 (17.5%)

	Total	138 (86.3%)	22 (13.7%)	160
1910+0937	Negative	129	3	132 (82.5%)
	Positive	8	20	28 (17.5%)
	Total	137 (85.6%)	23 (14.4%)	160
1718+0612	Negative	126	6	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	133 (83.1%)	27 (16.9%)	160
1718+0937	Negative	128	4	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	135 (84.4%)	25 (15.6%)	160
0092+0612	Negative	132	0	132 (82.5%)
	Positive	8	20	28 (17.5%)
	Total	140 (87.5%)	20 (12.5%)	160
0092+0937	Negative	132	0	132 (82.5%)
	Positive	8	20	28 (17.5%)
	Total	140 (87.5%)	20 (12.5%)	160
0612+0937	Negative	126	6	132 (82.5%)
	Positive	9	19	28 (17.5%)
	Total	135 (84.4%)	25 (15.6%)	160
1910+1718+0092	Negative	131	1	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	138 (86.3%)	22 (13.7%)	160
1910+1718+0612	Negative	130	2	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	137 (85.6%)	23 (14.4%)	160
1910+1718+0937	Negative	130	2	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	137 (85.6%)	23 (14.4%)	160
1910+0092+0612	Negative	130	2	132 (82.5%)

	Positive	7	21	28 (17.5%)
	Total	137 (85.6%)	23 (14.4%)	160
1910+0092+0937	Negative	130	2	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	137 (85.6%)	23 (14.4%)	160
1910+0612+0937	Negative	130	2	132 (82.5%)
	Positive	8	20	28 (17.5%)
	Total	138 (86.3%)	22 (13.7%)	160
1718+0092+0937	Negative	130	2	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	137 (85.6%)	23 (14.4%)	160
1718+0612+0937	Negative	127	5	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	134 (83.8%)	26 (16.2%)	160
0092+0612+0937	Negative	132	0	132 (82.5%)
	Positive	8	20	28 (17.5%)
	Total	140 (87.5%)	20 (12.5%)	160
All except 1718	Negative	130	2	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	137 (85.6%)	23 (14.4%)	160
All except 0092	Negative	130	2	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	137 (85.6%)	23 (14.4%)	160
All except 0612	Negative	131	1	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	138 (86.3%)	22 (13.7%)	160
All except 0937	Negative	131	1	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	138 (86.3%)	22 (13.7%)	160

All proteins	Negative	131	1	132 (82.5%)
	Positive	7	21	28 (17.5%)
	Total	138 (86.3%)	22 (13.7%)	160

Table S4: Statistical factors associated with the comparison between the indirect immunofluorescence antibody test (IFAT) and the recombinant protein-based ELISA for the detection of serum phase I IgG antibodies against C. burnetii. The statistical analysis was performed based on Binary logistic regression. For the combination of the proteins 1718+0092, 1718+0092+0612 and the combination of all the proteins without 1910 the analysis failed to produce any results since the Hosmer and Lemeshow test failed. Column B indicates the factor that should be multiplied with the value of each protein as detected following the ELISA testing. Column Constant indicates the value that should be subtracted following the multiplication. The name inside the brackets at the Exp(B) indicates the protein that the model kept following the analysis.

													Model build up			
Protein/combinations of proteins	**TPR**	**TNR**	**PPV**	**NPV**	**FPR**	**FNR**	**lr+**	**lr-**	**OR**	**Acc**	**Pe**	**κ**	**B**	**Exp(B)**		**Constant**
1910	0,714	0,970	0,833	0,941	0,030	0,286	23,571	0,295	80,000	0,925	0,728	0,725	3,398	29,912		-7,674
1718	0,750	0,939	0,724	0,947	0,061	0,250	12,375	0,266	46,500	0,906	0,707	0,680	2,009	7,453		-5,259
0092	0,714	1,000	1,000	0,943	0,000	0,286	0,000	0,286	0,000	0,950	0,744	0,805	2,674	11,495		-3,505
0612	0,679	0,962	0,792	0,934	0,038	0,321	17,914	0,334	53,622	0,913	0,728	0,679	1,776	5,904		-3,421
0937	0,429	0,970	0,750	0,889	0,030	0,571	14,143	0,589	24,000	0,875	0,760	0,479	2,238	9,377		-3,297
1910+1718	0,786	0,977	0,880	0,956	0,023	0,214	34,571	0,219	157,667	0,944	0,723	0,797	2,76	15,799	[1910]	-7,928
1910+0092	0,750	0,985	0,913	0,949	0,015	0,250	49,500	0,254	195,000	0,944	0,732	0,790	2,541	12,696	[1910]	-6,834
1910+0612	0,714	0,985	0,909	0,942	0,015	0,286	47,143	0,290	162,500	0,938	0,736	0,764	2,954	19,189	[1910]	-7,603
1910+0937	0,714	0,977	0,870	0,942	0,023	0,286	31,429	0,292	107,500	0,931	0,732	0,744	3,058	21,28	[1910]	-7,879
1718+0612	0,750	0,955	0,778	0,947	0,045	0,250	16,500	0,262	63,000	0,919	0,715	0,715	1,742	5,708	[1718]	-5,247
1718+0937	0,750	0,970	0,840	0,948	0,030	0,250	24,750	0,258	96,000	0,931	0,723	0,751	1,76	5,815	[1718]	-5,584
0092+0612	0,714	1,000	1,000	0,943	0,000	0,286	0,000	0,286	0,000	0,950	0,744	0,805	2,713	15,069	[0092]	-3,481
0092+0937	0,714	1,000	1,000	0,943	0,000	0,286	0,000	0,286	0,000	0,950	0,744	0,805	2,787	16,228	[0092]	-3,391
0612+0937	0,679	0,955	0,760	0,933	0,045	0,321	14,929	0,337	44,333	0,906	0,723	0,661	1,383	3,985	[0612]	-3,644

1910+1718+0092	0,750	0,992	0,955	0,949	0,008	0,250	99,000	0,252	393,000	0,950	0,736	0,811	2,169	8,749	[1910]	-7,091
1910+1718+0612	0,750	0,985	0,913	0,949	0,015	0,250	49,500	0,254	195,000	0,944	0,732	0,790	2,656	14,24	[1910]	-7,869
1910+1718+0937	0,750	0,985	0,913	0,949	0,015	0,250	49,500	0,254	195,000	0,944	0,732	0,790	2,597	13,424	[1910]	-8,278
1910+0092+0612	0,750	0,985	0,913	0,949	0,015	0,250	49,500	0,254	195,000	0,944	0,732	0,790	2,54	12,685	[1910]	-6,863
1910+0092+0937	0,750	0,985	0,913	0,949	0,015	0,250	49,500	0,254	195,000	0,944	0,732	0,790	2,545	12,739	[1910]	-7,002
1910+0612+0937	0,714	0,985	0,909	0,942	0,015	0,286	47,143	0,290	162,500	0,938	0,736	0,764	2,94	18,912	[1910]	-7,66
1718+0092+0937	0,750	0,985	0,913	0,949	0,015	0,250	49,500	0,254	195,000	0,944	0,732	0,790	1,225	3,405	[1718]	-4,547
1718+0612+0937	0,750	0,962	0,808	0,948	0,038	0,250	19,800	0,260	76,200	0,925	0,719	0,733	1,776	5,907	[1718]	-5,599
0092+0612+0937	0,714	1,000	1,000	0,943	0,000	0,286	0,000	0,286	0,000	0,950	0,744	0,805	2,774	16,027	[0092]	-3,392
All except 1718	0,750	0,985	0,913	0,949	0,015	0,250	49,500	0,254	195,000	0,944	0,732	0,790	2,545	12,748	[1910]	-6,997
All except 0092	0,750	0,985	0,913	0,949	0,015	0,250	49,500	0,254	195,000	0,944	0,732	0,790	2,602	13,492	[1910]	-8,196
All except 0612	0,750	0,992	0,955	0,949	0,008	0,250	99,000	0,252	393,000	0,950	0,736	0,811	2,209	9,106	[1910]	-7,369
All except 0937	0,750	0,992	0,955	0,949	0,008	0,250	99,000	0,252	393,000	0,950	0,736	0,811	2,103	8,194	[1910]	-6,882
All proteins	0,750	0,992	0,955	0,949	0,008	0,250	99,000	0,252	393,000	0,950	0,736	0,811	2,141	8,507	[1910]	-7,405

Table S5: All ELISA results about the first group of proteins studied for their ability to differentially diagnose Q fever disease.

	Patient ID	IgG	IgM	Phl IgG	Phll IgG	Other bacteria	IgG	IgM	Com1 (CBU_1910)	HspB (CBU_1718)	YbgF (CBU_0092)	OmpH (CBU_0612)	UPF0422 (CBU_0937)
Chronic	2265			61440	30720				2,2	2,24	0,2	0,26	0,28
	2052			4096	8192				3,62	3,53	3,76	2,28	0,75
	2181			16384	16384				3,63	3,49	3,1	2,19	2,93
	2316			32768	65536				3,62	3,45	3,6	2,36	0,85
Blood Donors	1983			0	0				0,61	0,16	0,27	0,39	0,49
	2762			0	0				0,92	2,49	0,28	0,3	0,68
	2764			0	0				1,01	0,29	0,13	0,16	0,36
	2768			0	0				0,71	0,33	0,23	0,28	0,45
	2804			0	0				1,03	1,62	0,33	0,52	0,93
	2733			0	0				1,34	0,58	0,16	0,4	0,58
	63691			4024	512				0,73	0,27	0,19	0,28	0,46
	64501			0	0				0,87	0,56	0,19	0,34	0,88
	12			0	0				1,02	0,35	0,18	0,2	0,41
	53601			0	0				1,12	0,42	0,24	0,34	0,61

	54653			0	0				0,59	0,45	0,18	0,23	0,38
	2			0	0				0,66	0,59	0,17	0,29	0,37
Other Pathogens	368					Chlamydia pneumoniae	1,8	0,31	0,73	0,4	0,23	0,38	0,26
	370					Chlamydia pneumoniae	1,71	0,16	1,9	1,25	0,11	0,59	0,43
	53425					Chlamydia pneumoniae	2,19	1,08	1,43	0,81	0,14	0,38	0,38
	54441					Chlamydia pneumoniae	1,48	0,5	0,75	0,38	0,14	0,28	0,22
	54197					Chlamydia pneumoniae	1,73	5,49	0,76	0,72	0,23	0,29	0,31
	282					Legionella	256	0	1,15	1,95	0,11	0,36	0,12
	53883					Legionella	1024	0	1,19	0,15	0,14	0,3	0,15
	365					Legionella	256	0	1,9	0,69	0,27	0,41	0,15
	367					Legionella	512	0	2,2	2,75	0,19	0,42	0,22
	376					Legionella	256	0	1,26	0,66	0,16	0,46	0,13
Other Pathogens	385					Legionella	256	0	0,95	0,67	0,19	0,42	0,11

	53924					Legionella			1,09	0,2	0,13	0,32	0,22
	1932			1024	1024	Chronic Burcellosis			1,08	0,58	0,25	0,53	0,88
	2219			0	240	Chronic Burcellosis			1,4	0,58	0,24	0,56	0,95
Rheumatoid Disease	SLE5W			0	0				0,74	1,501	0,438	1,348	0,515
	RA2M			0	0				0,373	2,511	0,118	2,518	0,855
	SLE1W			0	0				0,692	0,631	0,448	0,82	0,403
	SLE8W			0	1024				0,315	0,785	0,114	0,574	0,543
	SLE2W			0	0				0,502	1,589	0,514	1,397	0,525
	SLE3W			0	0				0,419	1,119	0,213	1,203	1,08
	SLE4W			0	0				0,375	1,624	0,347	1,007	0,826
	RA7W			0	4096				0,552	1,237	0,522	0,911	1,123
	RA10M			2048	2048				0,615	2,489	0,345	1,838	0,711
	RA1M			512	4096				0,48	1,119	0,292	1,559	0,536
	SLE6M			2048	8192				0,444	1,394	0,166	1,416	2,458
	SLE10M			512	2048				0,437	1,714	0,387	2,449	0,858

	RA3W			0	0				0,578	1,548	0,337	1,873	0,712
	RA6W			0	0				0,53	0,638	0,234	0,663	0,75
	RA9M			0	8192				0,743	0,679	0,46	0,606	0,316
	RA5W			0	512				0,521	0,409	0,32	0,818	2,118
	SLE9M			2048	8192				0,321	3,264	0,316	3,396	1,744
	RA8W			0	1024				0,438	1,208	0,322	1,165	0,753
	RA4W			0	0				0,53	3,349	0,575	3,482	1,514
	SLE7W			512	2048				0,447	2,171	0,234	2,138	1,105
	1392	7680	0	256	512				0,75	1,09	0,16	0,36	0,48
	1324	7680	50	256	1024				1,17	1,44	0,21	0,36	0,28
	2253			256	4096				1,33	0,36	0,17	0,33	0,15
	2264			512	4096				1,23	0,37	0,21	0,35	0,16
	2296			512	4096				0,71	0,3	0,2	0,35	0,18
	2402			512	4096				1	0,46	0,13	0,4	0,16
	2595			0	1024				0,78	0,39	0,14	0,36	0,39

Negative PhI	2451	3840	0	512	128				1,01	0,76	0,16	0,63	0,45
	2280	3840	50	128	512				0,57	0,27	0,12	0,32	0,28
	2249	7680	0	256	1024				1,18	0,23	0,19	0,26	0,18
	1285	3840	0	0	256				1,76	0,4	0,33	0,55	0,69
	83			1024	4096				1,96	2,52	0,28	0,58	0,58
	864	3840	25	0	0				0,56	0,72	0,24	0,32	0,52
	163			512	512				0,87	0,54	0,13	0,37	0,34
	195			256	1024				0,64	0,39	0,22	0,28	0,27
	239			128	2048				0,91	0,48	0,18	0,45	0,52
	16			256	512				0,69	0,34	0,18	0,39	0,9
	333			128	128				0,78	0,39	0,15	0,35	0,54
	40			256	512				0,5	0,14	0,24	0,24	0,2
	344			512	2048				0,73	0,18	0,59	0,24	0,24
	62			256	1024				0,56	0,22	0,65	0,26	0,43
	673			512	1024				0,74	0,16	0,47	0,28	0,26
	1152			512	2048				0,85	0,18	0,28	0,3	0,29

Negative PhI	84			256	512				0,86	0,19	0,17	0,32	0,13
	255			512	1024				0,45	0,14	0,08	0,18	0,11
	48			64	256				0,54	0,12	0,12	0,18	0,22
	1318	3840	0	256	1024				1,29	0,36	0,21	0,68	0,58
	1283	7680	0	256	1024				1,24	0,34	0,16	0,32	0,32
	1318			512	4096				1,78	0,39	0,14	0,58	0,44
	2095			512	8192				2	3,18	0,23	0,78	0,83
	2049			512	256				0,74	0,31	0,17	0,31	0,28
	2476	3840	0	512	1024				0,98	2,49	0,25	0,36	0,74
	46			256	4096				1,65	2,82	0,2	0,45	0,11
	100			128	128				2,75	2,8	0,33	0,4	0,43
	342			512	2048				1,86	3,1	0,43	0,48	0,11
	362			512	512				1,83	2,84	0,29	0,41	0,52
	2266			256	1024				0,64	0,34	0,17	0,23	0,29
	2252	3840	0	0	512				1,08	0,43	0,26	0,39	0,38
	2243	3840	0	0	256				0,5	0,42	0,25	0,4	0,45

	738	3840	0	0	128				0,86	1,13	0,23	0,31	0,39
	320			512	512				0,85	0,31	0,21	0,57	0,82
	935	3840	25	512	1024				0,93	1,65	0,7	0,4	0,5
	314			512	2048				0,63	0,18	0,1	0,16	0,4
	2440	3840	100	0	0				1,06	0,27	0,25	0,36	0,38
	1309	3840	0	512	1024				0,62	0,49	0,13	0,17	0,28
	2411	3840	0	128	512				0,67	0,88	0,22	0,71	0,26
	1106			512	1024				0,77	0,18	0,13	0,21	0,08
	708	3840	0	256	512				0,79	0,49	0,19	0,18	0,22
	776	3840	0	256	512				1,73	0,72	0,22	0,3	0,53
	1123			512	2048				2,2	0,75	0,29	0,45	0,42
	762	3840	0	256	512				0,95	0,43	0,16	0,17	0,25
	825	3840	0	512	1024				0,54	0,45	0,15	0,18	0,23
	56			512	2048				1,98	3,32	0,29	0,51	0,18
	2370	3840	0	0	256				0,2	0,08	0,1	0,1	0,14
Negative	2246	3840	0	512	1024				0,95	0,89	0,2	0,56	0,49

PhI	2376	3840	50	0	0				0,46	0,35	0,13	0,19	0,08
	2248	7680	0	256	1024				0,69	0,53	0,18	0,57	0,41
	2275	7680	0	128	512				0,68	0,36	0,24	0,59	0,65
	1393	3840	0	128	256				0,58	2,39	0,23	0,33	0,73
	256			512	512				1,01	0,63	0,91	0,49	0,88
	2401	7680	0	512	4096				0,59	0,77	0,19	0,33	0,55
At least one positive PhI	252			1024	2048				2,01	1,37	0,27	0,61	0,57
	270			64	1028				1,56	0,98	0,24	0,59	0,78
	855			256	1024				1,58	0,98	0,18	0,55	0,8
	1423			64	2048				1,47	1,52	0,18	0,56	0,45
	1887			512	2048				1,36	1,12	0,22	0,65	0,81
	1917			512	2048				1,57	0,93	0,19	0,67	0,32
	2211			512	2048				1,1	0,8	0,19	0,54	0,33
	1303			256	4096				1,13	0,69	0,19	0,21	0,15
	1394			1024	4096				1,45	0,75	0,21	0,3	0,26
	86			1024	512				1,11	0,75	0,24	0,29	0,3

	114			1024	512				1,09	0,62	0,22	0,32	0,68
	154			2048	512				1,16	0,49	0,27	0,28	0,15
	196			512	1024				2,27	0,65	0,35	0,34	0,31
	274			2048	1024				1,11	0,47	0,47	0,15	0,12
	82			2048	1024				1,1	0,34	0,37	0,18	0,41
	216			1024	1024				1,05	0,36	0,42	0,39	0,75
	399			1024	512				0,98	0,44	0,29	0,33	0,51
	661			2048	2024				1,09	0,38	0,28	0,25	0,52
	912			2048	2048				1,21	0,3	0,23	0,24	0,54
	2249			512	8192				0,62	0,22	0,28	0,34	0,18
	2280			1024	2048				0,78	0,23	0,18	0,36	0,49
	83			1024	4096				1,96	2,52	0,28	0,58	0,58
	306			1024	1024				0,71	0,15	0,2	0,21	0,12
	2099			4096	4096				3,4	3,19	0,41	1,41	0,3
	1898			1024	2048				0,7	0,28	0,2	0,35	0,34
	2011			1024	2048				0,62	0,21	0,19	0,31	0,32

At least one positive PhI	2078			256	4096				0,62	0,25	0,19	0,42	0,36
	2238			1024	2048				0,67	0,45	0,2	0,31	0,28
	2388			2048	8192				0,82	0,16	0,17	0,31	0,55
	1706			4096	2048				2,55	0,73	0,4	0,4	0,41
	708			256	512				0,79	0,49	0,19	0,18	0,22
	776			256	512				1,73	0,72	0,22	0,3	0,53
	276			1024	1024				1,2	0,41	0,24	0,31	0,53
	313			1024	1024				1,09	0,52	0,23	0,38	0,61
	65			64	512				1,36	0,59	0,28	0,47	0,75
	162			512	1024				1,23	1,8	0,17	0,41	0,64
	190			128	512				1,93	1,59	0,23	0,34	0,53
	202			1024	2048				1,9	1,99	0,18	0,33	0,44
	203			1024	1024				1,48	1,75	0,22	0,28	0,36
	289			2048	2048				2,27	0,5	0,25	0,37	0,94
	318			2048	2048				2,06	0,57	0,22	0,4	0,79
	148			1024	32768				1,95	3,1	2,6	1,29	0,63

At least one positive PhI	2076			4096	512				0,79	0,29	0,13	0,39	0,22
	1295			1024	4096				1,5	1,4	0,31	0,25	0,26
	85			4096	19384				2,06	2,18	0,23	0,64	0,68
	803			1024	2048				0,91	0,33	0,16	0,17	0,17
	1967			1024	128				0,86	0,25	0,17	0,46	0,51