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CONCISE ARTICLE

Identification of protein candidates for the serodiagnosis of Q fever endocarditis by an immunoproteomic approach

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Abstract Q fever is a worldwide zoonosis caused by *Coxiella burnetii* bacterium. Two clinical forms are present: acute Q fever and chronic disease, including endocarditis. Currently, the diagnosis of Q fever endocarditis is based on the detection of anti-phase I antibodies. The objective of the study was to identify candidate proteins for the serological diagnosis of endocarditis due to *C. burnetii*. The immunoreactivities of sera from 12 patients with *C. burnetii* infections, including the sera from patients with endocarditis and with the acute clinical form of Q fever, were compared with those of three control subjects who did not have Q fever. We identified 29 candidate antigenic proteins by mass spectrometry. Two proteins, arginine repressor and OmpH, were recognised exclusively by the sera of patients with Q fever endocarditis. These proteins are promising candidates for the development of serodiagnostic assays for Q fever endocarditis.

Abbreviations

IEF	Isoelectric focussing
COG	Cluster of orthologous groups
IE	Infective endocarditis

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Introduction

Coxiella burnetii is a strict intracellular bacterium responsible for Q fever in humans, livestock and other vertebrates. Reservoirs of infection include both wild and domestic mammals, birds and arthropods [1]. Cattle, goats and sheep are considered to be the primary reservoirs from which human infections originate [2]. In humans, the disease is generally acquired via the respiratory route by the inhalation of infectious aerosols and may be present in two forms: acute and chronic [1].

The most common acute form of Q fever is manifested in humans as atypical pneumoniae, self-limited febrile illnesses or granulomatous hepatitides. Infection can also be asymptomatic. The clinical expression of acute Q fever has already been described [3].

Persistent *C. burnetii* infection may lead to a chronic form of the disease with endocarditis, osteomyelitis or infected aortic aneurysms [4, 5]. Q fever endocarditis is often a severe disease associated with a long diagnostic delay. The positive blood culture is a gold standard for Q fever endocarditis diagnosis. However, sterile blood cultures are noted in one third of patients with infectious endocarditis [6]. Most often, the antibiotics treatment precedes blood culture; this holds true in 45–60% of cases of culture-negative endocarditis (CNE) [7]. The diagnosis is most often based upon the detection of vegetation on the cardiac valves using echography [8]. It has been shown that the sensitivity of *C. burnetii* endocarditis detection is possible in only 13% of Q fever cases [6, 9]. The lack of systematic serological testing for *C. burnetii* in CNE is the important limiting factor in the aetiological diagnosis of CNE in patients with infectious endocarditis reported in the literature [9, 10]. Many serological methods have been used to rapidly diagnose infection with *C. burnetii*; however,

ambiguous results are frequently obtained [6, 8]. The diagnosis based on serum antibody response to *C. burnetii* phase I and II is carried out only by specialised laboratories. In such situations, the diagnosis of Q fever endocarditis remains a diagnostic challenge.

The most reliable and commonly used methods are indirect immunofluorescence [11] and complement fixation [12]. Both *C. burnetii* Nine-Mile strain phase I and phase II are used as antigens. Antibodies to phase I and phase II have been detected in the IgG, IgM and IgA classes. Q fever endocarditis has been associated with very high titres of anti-phase I IgG antibodies. Cross-reactions however, may be a source of confusion when interpreting serological results [13]. The molecular detection of *C. burnetii* in blood and sera during illness is also performed [14].

The development of useful recombinant antigens could prove to be a step towards the development of novel approaches for detecting *C. burnetii* in clinical samples, and also for detecting new targets for serodiagnostics and vaccine development. Immunoproteomics is one method to identify such candidate proteins. Several proteomic studies have been reported recently, but none clearly addresses proteins for the diagnosis of Q fever endocarditis [15, 16]. A recent study reported efforts to identify specific protein markers for three clinical isolates of *C. burnetii* using a matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry [17]. Furthermore, an immunoproteomic approach [15] has been undertaken to characterise specific proteins from the developmental forms of small-cell variants/large-cell variants (SCV/LCV) of *C. burnetii* involved in protective immunity. A biphasic developmental cycle whereby highly resistant SCVs are generated from LCVs is considered to be fundamental to the virulence of *C. burnetii*. The identification of cell-form specific and common antigens provided new information for the development of subunit vaccines and new diagnostic tests.

In the present study, we focussed on the identification of serological markers for diagnosing Q fever endocarditis using an immunoproteomic approach combined with mass spectrometry.

Methods

Sera

Diagnosis of Q fever-positive patients and the selection of human sera

The diagnosis of patients with Q fever was achieved by serology [11]. The diagnosis of infective endocarditis (IE) was performed using modified Duke's criteria [18]. The sera from five patients suffering from acute Q fever, seven patients suffering from Q fever endocarditis and three healthy blood donors were selected for subsequent studies. They are all listed in Table 1 and the profiles of antibody response (IgG, IgM, IgA, phase I and phase II) for each patient is shown in Fig. 1.

Bacterial culture and purification

Strain Nine Mile RSA 493 (*C. burnetii*, COXBU, 227377, genus *Coxiella*) from the American Type Culture Collection (ATCC, USA) was propagated on Vero cell monolayers (ATCC CRL 1587). Minimal essential medium (MEM) (Invitrogen, Cergy-Pontoise, France) supplemented with 4% foetal bovine serum (Invitrogen, Cergy-Pontoise, France) and 1% L-glutamine (Invitrogen, Cergy-Pontoise, France) was used for cultivation. Infected cells were maintained in a 5% CO_2 atmosphere at 35°C. *C. burnetii* cells were harvested, pelletted, resuspended in MEM and purified on a discontinuous renographin gradient as described previously [19]. Purified bacteria were washed in PBS (at 10,000g, 10 min) and stored at –80°C until use. All purification steps were monitored by Gimenez staining [20].

Preparation of crude extracts for 2-D gel electrophoresis

We applied the methods earlier described by Renesto et al. [21], with some modifications. The *C. burnetii* pellet was resuspended in 5 ml of 5 mM Tris-HCl buffer (pH 7.6) and lysed by two passages through a Constant Cell Disruption System (2 kBar). Cell debris and unbroken cells were discarded following a centrifugation cycle at 5,600g for 20 min at 4°C. The supernatant was transferred into a new tube and ultracentrifuged (100,000g for 2 h at 4°C in a Beckman MLS-50 rotor). After washing in 5 mM Tris-HCl, pH 7.6, the membrane-enriched pellet was resuspended in rehydration solution (7 M urea, 2 M thiourea, 4% (w/v) CHAPS) and stored at –80°C. Proteins were precipitated using the PlusOne 2-D Clean-Up Kit (GE Healthcare, Chalfont St. Giles, UK) and suspended again in rehydration solution. To conserve the restricted amounts of proteins, we used Immobiline™ DryStrips, pH 3–10 (GE Healthcare, Chalfont St. Giles, UK) of two different lengths, 7 or 18 cm, depending on the subsequent application. Immunoblotting was performed on 7-cm strips, while spots on 18-cm strips were identified by mass spectrometry. Strips were rehydrated overnight with the 20 µg/7-cm strip and the 150 ug/18-cm strip of proteins in rehydration solution supplemented with 0.5% (v/v) IPG buffer (pH 4–7) (GE Healthcare, Chalfont St. Giles, UK). The isoelectric focussing (IEF) was carried out according to the manufacturer's instructions (Ettan IPGphor II, GE Healthcare, Chalfont St. Giles, UK). Prior to gel electrophoresis in the second dimension, the strips were equilibrated as previously described [21]. The strips were

Table 1 Human sera from patients diagnosed with Q fever and healthy blood donors

N° serum	Figure 3 part	Type of Q fever disease	Sex	Age	Phase I	Phase II	Age, mean ± SD
1	A	Acute/pneumonia	M	40	IgG 200 IgM 800 IgA 400	IgG 400 IgM 1600 IgA 800	
2	B	Acute/hepatitis	M	19	IgG 200 IgM 400 IgA 50	IgG 400 IgM 800 IgA 100	
3	C	Acute/pneumonia	M	39	IgG 50 IgM 800 IgA 0	IgG 100 IgM 1600 IgA 0	36.8 ± 11.95
4		Acute/ lymphadenopathy	M	52	IgG 50 IgM 1600 IgA 0	IgG 100 IgM 3200 IgA 0	
5		Acute/pneumonia	M	34	IgG 100 IgM 800 IgA 25	IgG 200 IgM 1600 IgA 50	
6	G	Endocarditis	F	69	IgG 6400 IgM 0 IgA 800	IgG 12800 IgM 0 IgA 1600	
7	F	Endocarditis	M	57	IgG 12800 IgM 400 IgA 3200	IgG 25600 IgM 800 IgA 6400	69.80 ± 17.82 (M)
8		Endocarditis	F	75	IgG 6400 IgM 100 IgA 1600	IgG 12800 IgM 200 IgA 3200	72.0 ± 3.0 (F)
9		Endocarditis	M	51	IgG 6400 IgM 800 IgA 1600	IgG 12800 IgM 1600 IgA 3200	
10	E	Endocarditis	M	86	IgG 800 IgM 0 IgA 0	IgG 1600 IgM 0 IgA 0	
11		Endocarditis	M	83	IgG 1600 IgM 0 IgA 100	IgG 3200 IgM 0 IgA 200	
12		Endocarditis	F	72	IgG 51200 IgM 0 IgA 400	IgG 102400 IgM 0 IgA 800	
13		Healthy blood donor	No data available	No data available	No data available	No data available	
14	D	Healthy blood donor	No data available	No data available	No data available	No data available	
15	H	Healthy blood donor	No data available	No data available	No data available	No data available	

then embedded in 0.5% agarose and the proteins were resolved by electrophoresis through a 10% acrylamide gel (Ettan™ DALT, GE Healthcare, Chalfont St. Giles, UK) for the 18-cm strips and an 11.25% acrylamide gel for the 7-cm strips (Bio-Rad Protean Mini xi chamber), as described by the manufacturer. Electrophoresis was performed until the bromophenol blue dye front reached the bottom of the gel. For protein identification following electrophoresis, the gels were silver-stained [22] and digitised by transmission scanning (ImageScanner, GE Healthcare, Chalfont St. Giles, UK).

Immunoblot assay

For Western blotting, the polyacrylamide 2-D gels were transferred onto a nitrocellulose membrane in a transblot cell (Bio-Rad) at 100 V for 1 h 30 min in an ice bath. Membranes were then blocked in TBST (Tris-HCl 50 mM (Invitrogen Cergy-Pontoise, France), NaCl 250 mM supplemented with 0.5% Triton X-100) and 5% non-fat dried milk overnight. Then, they were incubated with the human sera as described in Table 1 in solution TBST and 5% non-fat dried milk (dilution of 1:500). Following 1 h incubation,

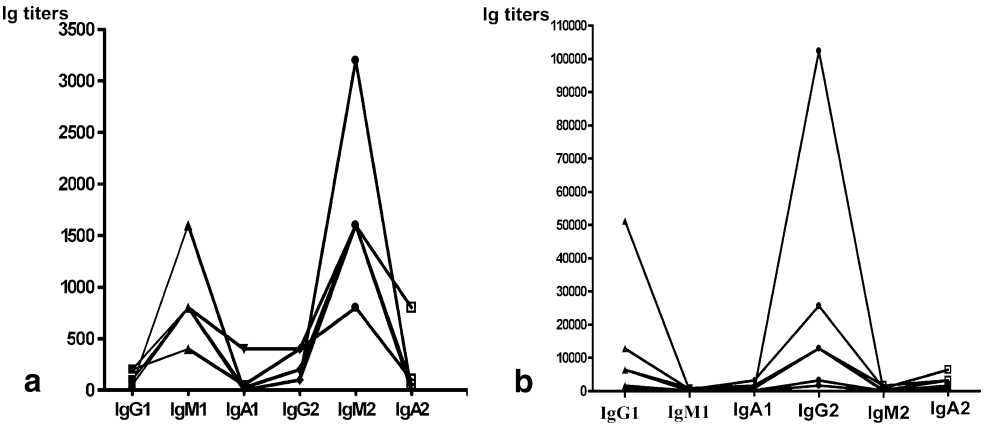

Fig. 1 Seroprevalence of antibodies phase I (IgG1, IgM1 and IgA1) and phase II (IgG2, IgM2 and IgA2) to *Coxiella burnetii* among the sera from patients suffering from Q fever. **a** Acute Q fever. **b** Endocarditis due to *C. burnetii*

the membranes were washed three times in TBST, incubated with peroxidase-conjugated immunoglobulin (goat anti-human Ig (H+L) 1:1000 (Southern Biotechnology, Birmingham, Alabama, USA). Each membrane was treated with the secondary antibody and washed three times, as previously described. Detection was carried out using a commercially available chemiluminescence kit (ECL™ Western Blotting Analysis System, GE Healthcare). The membranes were exposed to Hyperfilm™ ECL (GE Healthcare, Chalfont St. Giles, UK) and subsequently developed using an automated film processor (Hyperprocessor™, GE Healthcare, Chalfont St. Giles, UK).

Standard software was used for the analyses of the stained 2-D gels and blot images (ImageMaster™ 2D Platinum version 6.0, GE Healthcare, Chalfont St. Giles, UK). For 2-D gels, these analyses included spot detection, matching and the estimation of isoelectric point (p*I*) and molecular weight (M_r). For each individual, immunoblots were performed at least twice. Immune profiles of the spots were analysed when two independent assays had produced identical patterns. We superimposed the immunoblot images over those on gels using the subset of spots (CBU_0937), which were recognised by the majority of the subjects and, consequently, was used for internal calibration. Thus, this approach permits the direct determination of the position for prominent spots on the 2D silver-stained gel and manually excised for consequent MALDI-TOF mass spectrometry analysis.

Digestion of proteins and MALDI-TOF mass spectrometry analysis

Protein spots excised from silver-stained gels were destained and subjected to in-gel digestion with trypsin (sequencing-grade modified porcine trypsin, Promega, Madison, WI, USA) [23]. Tryptic peptides were then extracted from the gel by successive treatment with 80% acetonitrile in 0.2% trifluoroacetic acid (TFA). Extracts were dried at ambient room temperature. Peptides were co-crystallised onto the MALDI target with a matrix solution of α-cyano-4-hydroxycinnamic acid in water/acetonitrile (1:1) with 0.5% TFA deposed two times with a volume of 0.5 µl. Mass analyses were performed with a MALDI-TOF/TOF Bruker Ultraflex II spectrometer (Bruker Daltonics, Wissembourg, France). The mass spectra were internally calibrated using autolytic peptides from trypsin.

To identify the proteins, the peptide mass lists were matched against databases proposed by the search engine Mascot (Matrix Science Ltd., London, UK), which is available at http://www.matrixscience.com/. In general, proteins with the highest sequence coverage and Mascot score were selected as candidate antigens [24].

Bioinformatics

We used HMMTOP 2.0, which is freely available at http://www.enzim.hu/hmmtop, to predict both the localisation of helical transmembrane segments and the topology of the identified proteins [25]. We classified the identified proteins into clusters of orthologous groups (COGs) functional categories according to the public database (http://www.ncbi.nlm.nih.gov/COG/). These are shown in Table 2.

Results

Western blot

The purification procedure for *C. burnetii* allowed the resolution of 250 proteins as determined by the software (ImageMaster™ 2D Platinium version 6.0, GE Healthcare, Chalfont St. Giles, UK). Spots of varying intensities with molecular masses ranging from 10 to 100 kDa were visualised on 7- and/or 18-cm gels, within a pH range from 3 to 10 (Fig. 2), respectively. Most of the proteins clustered within an acidic pH range of 4–7.

As illustrated in the shown examples of immunoblot of 2-D gel-separated antigens with human sera (Fig. 3), the antigenic recognition profiles obtained with the serum of

Table 2 Identification of Q fever discriminatory proteins

Spot no.	Identification	Locus	Locus tag	NCBI no.*	*MW*[#]	P*i*["]	Mascot score	E-value	Queries matched	% coverage	COG	Reference
Cb 1	Hypothetical protein CBU_0092	–	CBU_0092	gi|29540714	32.07	5.49	135	37	7	34%	COG1729S	
Cb 2	Protein mraZ	mraZ	CBU_0115	gi|29540735	17.39	5.29	106	2.1e−05	10	78%	COG2001S	
Cb 3	Translation elongation factor Tu	tuf-1	CBU_0223	gi|29540837	32.75	4.82	175	4.8e−12	21	70%	COG0050J	[49]
Cb 4	Translation elongation factor G	fusA	CBU_0235	gi|29540849	77.91	5.07	98	0.00023	19	28%	COG0480J	
Cb 5	Translation elongation factor Tu	tuf-2	CBU_0236	gi|29540850	43.	5.32	233	7.6e−18	27	74%	COG0050J	[15]
Cb 6	DNA-directed RNA polymerase alpha subunit	rpoA	CBU_0263	gi|29540877	35.71	5.61	149	1.9e−09	17	63%	COG0202K	
Cb 7	Single-strand binding protein	Ssb	CBU_0271	gi|29540885	17.43	5.61	59	1.7	6	37%	COG0629L	
Cb 8	Heat shock protein 90	htpG	CBU_0309	gi|29540922	72.84	5.2	93	0.00076	13	29%	COG0326O	[15]
Cb 9	Cytosol aminopeptidase family protein	–	CBU_0572	gi|29541173	51.03	5.56	82	0.012	9	25%	–	
Cb 10	Arginine repressor	argR	CBU_0480	gi|29541086	17.77	9.47	94	0.0004	8	56%	COG1438K	
Cb 11	Inorganic pyrophosphatase	Ppa	CBU_0628	gi|29541229	19.76	5.2	65	0.3	7	26%	COG0221C	
Cb 12	Hypothetical protein CBU_0632	–	CBU_0632	gi|29541233	11.94	4.69	61	1.1	6	67%	COG3461S	
Cb 13	6,7-dimethyl-8-ribityllumazine synthase	ribH	CBU_0648	gi|29541249	16.76	6.74	63	0.071	6	39%	–	[15]
Cb 15	Hypothetical protein CBU_0937	–	CBU_0937	gi|29541524	51.58	8.99	120	1.5e−06	16	48%	–	
Cb 16	Hypothetical protein[†] CBU_0952	–	CBU_0952	gi|29541535	25.94	8.67	110	1.5e−05	14	52%	–	[15]
Cb 17	Chaperone protein dnaK	dnaK	CBU_1290	gi|29541858	70.83	5.14	100	0.00016	14	23%	COG0443O	[49]
Cb 18	Transcription elongation factor NusA	nusA	CBU_1433	gi|29541992	56.41	4.59	180	1.7e−12	18	37%	COG0195K	[15]
Cb 19	Antioxidant, AhpC/Tsa family[†]	–	CBU_1706	gi|29542264	22.01	5.08	118	2.4e−06	13	64%	COG0450O	[16]
Cb 20	Chaperonin, 60 kDa	groEL	CBU_1718	gi|29542276	58.36	5.14	370	1.5e−31	40	66%	COG0459O	[15, 48, 49]
Cb 21	Co-chaperonin GroES	groES	CBU_1719	gi|29542277	10.50	5.18	107	3e−05	11	88%	COG0234O	[49]
Cb 22	ABC transporter, ATP-binding protein[†]	–	CBU_1770	gi|29542327	48.21	4.85	72	0.1	9	25%	COG1116P COG4175E COG4754S	
Cb 23	Hypothetical protein CBU_1789	–	CBU_1789	gi|29542346	32.22	4.95	61	1.2	7	28%	COG3386G	
Cb 24	Outer membrane protein[†]	Com1	CBU_1910	gi|29542467	27.72	9.23	65	0.44	9	40%	COG1651O	[48]
Cb 25	ATP synthase subunit B	atpD	CBU_1945	gi|29542501	50.46	5.01	137	3.4e−08	14	41%	COG0055C	
Cb 27	Glyceraldehyde 3-phosphate dehydrogenase, type I	Gap	CBU_1783	gi|29542340	36.47	5.93	89	0.0019	9	33%	COG0057G	
Cb 28	30S ribosomal protein S1	rpsA	CBU_0528	gi|29541131	62.20	5.28	103	8.5e−05	12	27%	COG0539J	[15]
Cb 29	3-deoxy-manno-octulosonate cytidylyltransferase	kdsB	CBU_0479	gi|29541085	28.29	5.01	88	0.0027	8	44%	COG1212M	[16]
Cb 30	Hypothetical protein	–	CBU_2029	gi|29542582	24.40	9.64	69	0.11	8	30%	–	
Cb 30	ompA-like transmembrane domain protein[†]	–	CBU_0307	gi|29540920	24.89	9.91	54	3.7	6	28%	–	
Cb 30	ompA-like transmembrane domain protein[†]	–	CBU_1260	gi|29541830	26.24	9.61	44	36	5	24%	–	
Cb 31	Outer membrane protein OmpH, putative†	–	CBU_0612	gi|29541213	18.82	9.71	43	49	5	32%	COG2825M	[16]

*Accession number according to NCBI
[#] Theoretical molecular weight
["] Theoretical isoelectric point
[†] Potential transmembrane protein

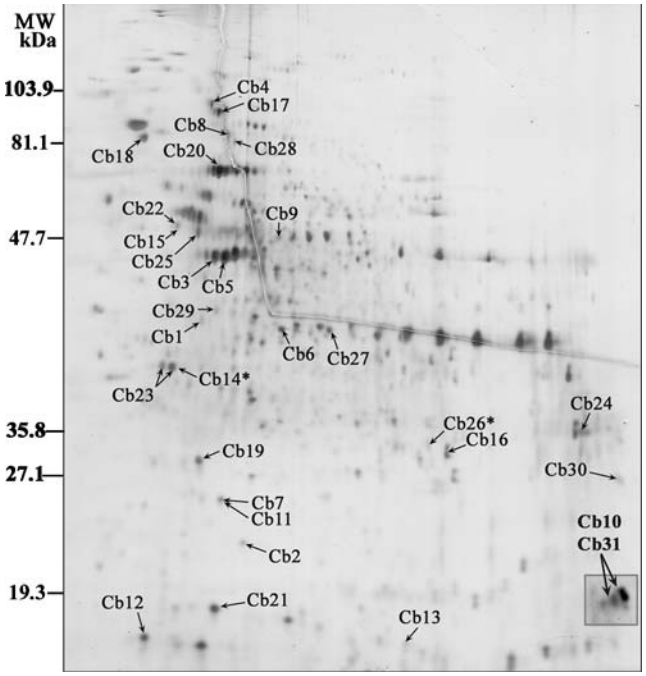

Fig. 2 Two-dimensional reference map of the whole-cell extract from *C. burnetii*. The proteins of *C. burnetii* (strain Nine Mile) were resolved by isoelectric focussing (IEF) and separated across the pH range 3–10 (18 cm), 10% acrylamide. The protein spots were visualised with silver nitrate staining. The *numbered arrows* indicate "discriminate antigens" of *C. burnetii*. The protein spots identified by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) are annotated (e.g. Cb1) and listed in Table 2. The magnified region on the right side of the gel shows the most discriminate spots for Q fever endocarditis patients, i.e. Cb10 identified as arginine repressor and Cb 31 identified as OmpH. The molecular weight marker is indicated on the left side of the gel

patients with acute Q fever (A, B, C) differed from those with endocarditis (E, F, G). The main distinction observed was the recognition of a significant antigenic protein spot in a low molecular weight range (17-, 18-kDa and p*I* about 10) that was not detected by the reaction with the sera of patients with the acute form of Q fever. The antigenic proteins were identified from the corresponding silver-stained gel. All reactive spots were identified (Table 2).

The immunoblots obtained with the sera of healthy blood donors (D, H), included in this study as a control group, have shown no cross-reactivity to *C. burnetii* proteins.

Identification of antigenic *C. burnetii* proteins

The most intense antigenic protein spots detected after silver staining were excised from seven different gels (five pH 3–10, 7-cm gels, and two pH 3–10, 18-cm gels), and were subject to analysis by MALDI-TOF mass spectrometry (Fig. 2). Twenty-nine proteins were successfully identified. Eight of them are potential transmembrane proteins. All proteins labelled in the gel (Fig. 2) and listed in Table 2 were repeatedly identified based on eight independent sets of data mapping to the same ORF. Two protein spots, nos. 14 and 26, were only identified in one gel, and, therefore, have been excluded from the table. The two spots that made a visible difference between the two major clinical manifestations of Q fever (Fig. 2, magnified square) were identified as an outer membrane protein, OmpH, and arginine repressor (Table 2). Several spots resulted in the same protein being identified, suggesting the presence of numerous isoforms: e.g. chaperonin, 60 kDa, CBU_1718; conserved hypothetical protein, CBU_0937; translation elongation factor Tu, CBU_0223.

The other identified proteins belonged to various COG categories (Table 2).

Discussion

The aim of this study was to find antigenic candidates for the development of serodiagnostic tests for Q fever endocarditis. We have successfully identified two protein candidates, namely, arginine repressor and OmpH. They were found to be exclusively antigenic with the sera obtained from patients suffering from chronic Q fever endocarditis.

These data have raised an interesting possibility of using these antigens to develop a serological test for the diagnosis of Q fever endocarditis. Most of the 27 remaining proteins identified in this study were antigenic with both sets of sera, acute and chronic. However, they might enable the serodiagnosis of both acute and chronic Q fever.

We are aware of several technical limitations in this study. The first involves matching protein spots with immunoblots following 2-D electrophoresis. Slight gel-to-gel variations were sometimes observed, but rigorous spot selection was applied and several gels were used for identification by MS. The second is related to the superior detection provided by the ECL reaction compared with silver staining. As a result of this, certain spots appeared as clustered on Western blots compared with silver-stained gels (Figs. 2 and 3). The last limitation results from the small cohort of patients included in this study.

The role of arginine repressor in transcriptional regulation in *C. burnetii* has yet to be completely elucidated. It a major component of biosynthetic pathways [26, 27] that regulates the initiation of transcription [28].

The genetic control of arginine-metabolising enzymes in many bacteria is achieved through the arginine repressor (ArgR) [29–33], which responds to intracellular levels of L-arginine. A content of arginine is influenced by arginine repressor. Bioinformatics analysis has revealed putative homologues of ArgR in many species [34–36]. Existence of

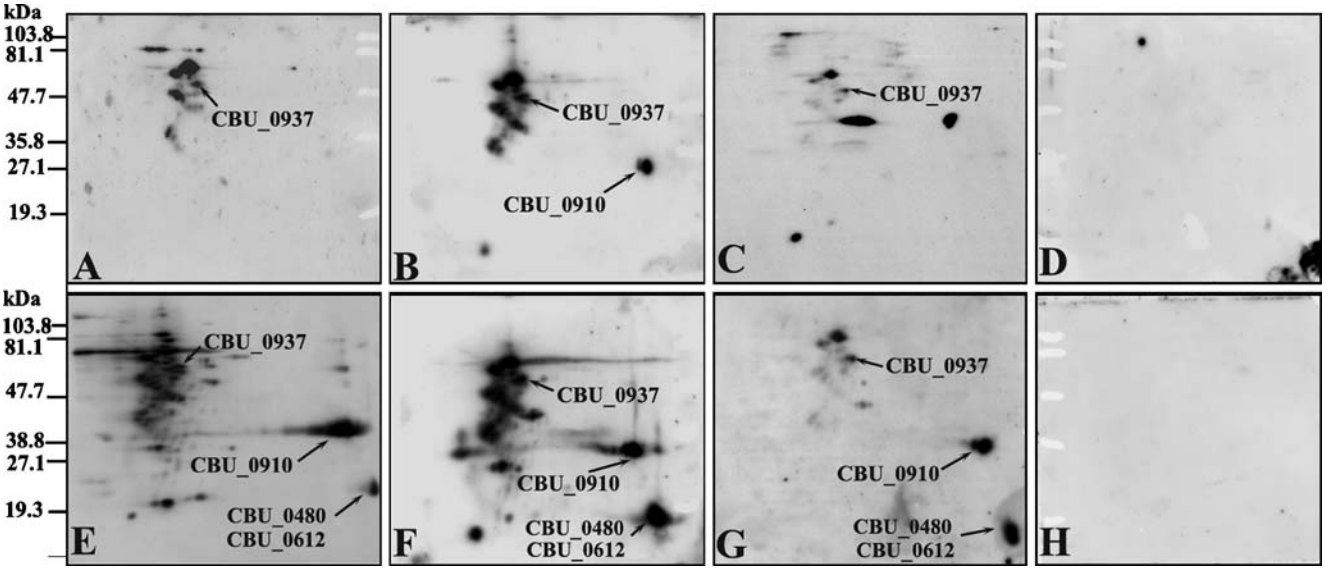

Fig. 3 Example of 2-D immunoblots profile obtained with the sera from *C. burnetii*-infected patients and healthy blood donors. The 2-D immunoblots probed with: (A, B, C) sera from the patients with acute Q fever; (E, F, G) sera from the patients with Q fever endocarditis; (D, H) sera from healthy blood donors. The human sera were diluted 1:500. All spots were subjected to MALDI-TOF identification. The *arrows* indicate the corresponding antigenic spots identified by MALDI-TOF: CBU_0937 used for internal calibration, and spots of interest: CBU_0480, CBU_0612 recognised by sera with Q fever endocarditis, CBU_0910 recognised by both sera with acute and chronic Q fever

the two types of argR alleles in natural population and the selective advantage of each type under different conditions was described and provided evidence for the evolutionary history of argR regulation by *Escherichia coli* [37]. The strategy of regulation may depend on the nature of the environment. However, the role of argR in the pathogenesis of endocarditis is not known.

OmpH, a cell wall/membrane/envelope biogenesis-associated protein, is an outer membrane protein widely studied in other bacteria and is known to be an excellent target for human antibodies [38, 39]. It may be an important factor for adhesion to host cells, with chaperonin-like activity, a protein important for the biosynthesis of the outer membrane. Antibody specificity for the corresponding antigen was already found in a comparable study for *Chlamydia pneumoniae* [40]. OmpH has been suggested as a potential candidate for vaccine development [40].

Some of the proteins identified in this study belong to the same cluster of orthologous groups as the two main proteins of interest to us (Table 2, COG K and/or M). They have been found to be discriminative in other immunoproteomic studies [41, 42], such as N utilisation transmembrane substance protein A (NusA) (Cb 18, CBU_1433) and DNA-directed RNA polymerase alpha subunit (Cb 6, CBU_0263). Both are able to modulate transcription [43]. We have identified the protein 3-deoxy-D-manno-octulosonate cytidylyltransferase protein (CMP-KDO synthase) as being antigenic (Cb 29, CBU_0479). CMP/KDO synthase inhibitors were described as a crucial factor for reducing the virulence of Gram-negative bacteria. Attempts have been made to find a potent inhibitor of 3-deoxy-D-manno-octulosonate cytidylyltransferase (CMP-KDO synthase) capable of inhibiting the growth of Gram-negative bacteria by interfering with the biosynthesis of lipopolysaccharide (LPS) [44]. The interaction of the bacterial agent with dendritic cells (DC) is dependent on the length of the LPS; the virulent form of *C. burnetii* Nine Mile is associated with full-length LPS, whereas the truncated form is associated with the avirulent form [45]. This LPS length-dependence has been postulated as a critical component of both innate and adaptive immunity of this bacteria [46, 47].

We assigned the proteins identified using the data we obtained into several functional categories (Table 2). Many of the proteins identified are known to be involved in *C. burnetii* developmental processing. Some of the identified proteins have been detected in previous studies using proteomic approaches [17, 48, 49]. We found several protein candidates that match those from these previous studies (Table 2), such as N utilisation substance protein A (Cb 18), which is able to modulate transcription; ribosomal protein S1 (Cb 28), which belongs to the large order of ribosomal proteins and plays an important role in translation system; hypothetical protein (Cb 16); heat shock proteins HtpG (Cb 8) and GroEL (Cb 20) destined for translocation through biological membranes; or elongation factor-Tu (Cb 5), one of the major proteins of *C. burnetii*, an essential component of the translational machinery of the cell. Com1 outer membrane protein (Cb 24), which is

known to be preferentially exposed on the surface of *C. burnetii*, and the gene encoding this protein, has been through trials as a genetic marker to distinguish among isolates [50]. These were all immunorecognised by the human sera in our study, except the hypothetical protein (Cb 16).

However, the objective of all of these proteomic studies was not exactly in line with the aim of our study. We were mostly interested in protein spots associated exclusively with chronic Q fever. We have successfully identified two targets which we believe to be the most suitable for developing Q fever endocarditis serodiagnostic assays, namely, arginine repressor (Cb 10, CBU_0480) and/or outer membrane protein OmpH (Cb 31, CBU_0612). Indeed, the development of endocarditis in patients with previous valvular lesions and suffering from Q fever is a major issue [51]. As a matter of fact, 30 to 50% of such patients will develop a chronic infection if untreated [52]. On the other hand, the duration of preventive treatment is long (one year), and may be hampered by adverse effects. Currently, repeating serology is the only possible strategy to detect an early valvular fixation [53]. Therefore, the raising of antibodies to these protein candidates may prove to be an efficient tool to detect an evolution towards chronic infection.

Conclusion

The combination of 2-D gel electrophoresis, immunoblotting and matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry allowed us to identify 29 candidate proteins for Q fever serology development. Two of them, OmpH and arginine repressor, were specifically associated with Q fever endocarditis. The next step will be to express these proteins in order to design a test that may be useful for detecting the evolution of chronic infection due to *Coxiella burnetii*.

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Conflicts of interest We declare that the authors have no conflicts of interest.

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