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Characterization of antigens for Q fever serodiagnostics

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Summary. – The aim of this study was to identify candidate proteins for serodiagnostics of Q fever by monoclonal antibodies (MAbs), and to clone, express, and purify the selected proteins for use as antigens in ELISA. The reactivity of three MAbs to *Coxiella burnetii* (*C. b.*) Nine Mile strain and one MAb to Priscilla strain was tested using SDS-PAGE, 2-D gel electrophoresis, immunoblot analysis, and mass spectrometry. Three immunoreactive Q fever-specific proteins discriminated by MAbs, namely the CBU_0937 protein, outer membrane Com1 (CBU_1910) protein, and elongation factor Tu (CBU_0236) were identified. Successful PCR-amplification, cloning, expression, and purification of the recombinant proteins Com1 and CBU_0937 allowed their use for the screening of sera from patients with Q fever endocarditis (18) or acute Q fever (16) in ELISA. The recombinant protein CBU_0937 with unknown biological function proved to be a more applicable diagnostic tool for Q fever ELISA as compared to the Com1 protein.

Keywords: Coxiella burnetii; monoclonal antibodies; 2-D electrophoresis; immunoblot analysis; recombinant protein; ELISA

Introduction

Coxiella burnetii is a Gram-negative obligate intracellular bacterium that is the causative agent of Q fever (Maurin and Raoult, 1999). The Q fever is a zoonosis transmitted mainly by inhalation route (Maurin and Raoult, 1999). In humans, the disease is generally acquired *via* the respiratory tract by the inhalation of infectious aerosol and may be present in two forms, acute and chronic (Maurin and Raoult, 1999). The most common acute form of Q fever is manifested as atypical pneumonia, self-limited febrile illness, or granulomatous hepatitis (Raoult, 2002). Persistent *C. b.* infection may lead to a chronic form of disease with the most common culture-negative endocarditis (CNE) (Maurin and Raoult, 1999; Raoult *et al.*, 2000). Q fever infective endocarditis (IE) is often a severe disease associated with a long diagnostic delay based upon the detection of vegetation on the cardiac valves using echography (Houpikian *et al.*, 2002; Raoult *et al.*, 1988). The lack of systematic serological testing for *C. b.* in CNE is the important limiting factor in etiological diagnosis of the CNE in patients with IE. The detection is possible only in 13% of Q fever cases (Raoult *et al.*, 2000).

Standard diagnostic schemes for both the Q fever IE and acute form of disease have been developed to improve the sensitivity and specificity (Raoult *et al.*, 2000; Fournier *et al.*, 1998; Fenollar *et al.*, 2004; Li *et al.*, 2000). However, despite advances in the diagnostic methods (Fenollar, 2004; Brouqui, 2006), a major aim to define a specific marker matching Q fever disease evolution remained open (Sekeyová *et al.*, 2009).

The aim of this study was (i) to identify candidate proteins for serodiagnostics of Q fever by three MAbs to *C. b.* Nine Mile strain, and one MAb to *C. b.* Priscilla strain, (ii)

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Abbreviations: *C. b.* = Coxiella burnetii; CNE = culture-negative endocarditis; IE = infective endocarditis; IF = immunofluorescence; MAb (s) = monoclonal antibody(ies); M_r = relative molecular mass; NPV = negative predictive value; NRL = negative likelihood ratio; PPV = predictive positive value; PRL = positive likelihood ratio; Se = sensitivity; Sp = specificity

to clone, express, and purify the selected recombinant proteins CBU_0937 and Com1 (CBU_1910) for use as antigens in ELISA, and (iii) to screen the sera from patients with Q fever IE and acute Q fever, respectively, in ELISA with the recombinant proteins.

Materials and Methods

Monoclonal antibodies (1-4-H) directed to strain Priscilla (Sekeyová *et al.*, 1995, 1996) and (2-13-1, 3-3-4, and 8-2-2) to Nine Mile strain (Sekeyová *et al.*, 1991) applied in this study were prepared as previously described (Kohler *et al.*, 1975).

Polyclonal antibody. The rabbits were immunized with *C. b.* strain Nine Mile intraperitoneally (Kazár *et al.,* 1978; Marrie and Raoult, 2002.)

Coxiella burnetii. Strain Nine Mile RSA 493 from the American Type Culture Collection (ATCC) was propagated on Vero cells as described before (Renesto *et al.*, 2005). All purification steps were monitored by staining (Gimenez *et al.*, 1964).

Patient sera. The diagnosis of IE caused by *C. b.* was performed using modified Duke's criteria (Li *et al.*, 2000) with serology as a major criterion (Fournier *et al.*, 1998). Sixteen Q fever acute sera with immunofluorescence (IF) titres ranging from 100 to 1,600 and 200 to 3,200 (IgG and IgM, respectively, phase I), 100 to 3,200 (IgG, phase II), 100 to 6,400 (IgM, phase II), and 18 Q fever IE sera with titres 200 to 25,600 (IgG phase I), 0 to 800 (IgM, phase I), 1,600 to 25,600 (IgG phase II), 0 to 3,200 (IgM, phase II) tested at the Unité des Rickettsies (Marseille, France) were included in this study. A control group included 14 IF negative human sera from anonymous healthy blood donors. Table 1 shows the main characteristics of individuals included in ELISA.

2-D electrophoresis. Protein samples were treated as previously described (Renesto *et al.*, 2005a,b). The Immobiline™ DryStrips, pH 3-10 (GE Healthcare) were rehydrated overnight with 20 µg/7 cm strip of proteins in rehydration solution. Isoelectric focusing was carried out according to the manufacturer's instructions (Ettan IPGphor II, GE Healthcare). Proteins were resolved by 2-D electrophoresis through 11.25% acrylamide gel, (Bio-Rad Protean Mini chamber) as described by the manufacturer. The gels were silver-stained (Nesterenko *et al.*, 1994) and digitized by transmission scanning (ImageScanner, GE Healthcare).

SDS-PAGE. All assays were carried out according to Cleveland *et al.* (1977) and Towbin *et al.* (1979) and stained same way as 2-D electrophoresis gels.

Digestion and MALDI-TOF mass spectrometry analysis. Protein spots excised from gels were subjected to in-gel digestion with sequencing grade modified porcine trypsin (Promega) as previously described (Shevchenko *et al.*, 1996). The mass analyses were performed with a MALDI-TOF/TOF Bruker Ultraflex II spectrometer. The available database Mascot at http://www.matrixscience.com/ was used for the identification of proteins.

Immunoblot assay. The SDS-PAGE and 2-D gels were processed as previously described (Renesto *et al.*, 2005a; Cleveland *et al.*, 1977; Towbin *et al.*, 1979; Renesto *et al.*, 2005b) with some modification in composition of buffer TBST (Tris-HCl 50 mmol/l, NaCl 250 mmol/l, 0.5% Triton X-100). Dilutions of MAbs (1:100), rabbit immune serum (1:5,000) and secondary peroxidase-conjugated immunoglobulin (1:1,000) were made in TBST and 5% non-fat dried milk. Detection was carried out using a chemiluminiscence kit (ECL™ Western Blotting Analysis System) and developed using an automated film processor.

Table 1. Characteristics of Q fever patient sera tested by ELISA

Characteristics	Patients		Control group
	Q fever IE	Q fever acute	Healthy donors
Total number	18	16	14
Male	12	11	N/A
Female	6	5	N/A
Age (years)	54.0 ± 19	44.7 ± 33.3	N/A

Plasmid constructs. DNA of *C. b.,* strain Nine Mile was extracted using commercially available kit (Qiagen). Primer pairs used to amplify the DNA fragment containing the *C. b.* gene Com1 were: F 5'-CGCCCCCTCTCAATCCATGGTTTCTCCTCAACAAG-3' and R 5'-CGTATCAGTGAGGATTTGAGCTCTTTCTACCCG GTC-3'. The gene fragment was cloned into the expression vector pIVEX 2.3d (Roche) at *Nco*I and *Sac*I sites (Sambrook *et al.*, 1989). Cloning was verified by sequencing of plasmid using T7 promoter and T7 terminator universal primers and by restriction mapping of the plasmid (Qiagen plasmid purification kit) and subsequent analysis on agarose gel.

The gene CBU_0937 was amplified into recombination sites Attb1/Attb2 (Gateway, Invitrogen) using primers: F 5'-GGGGACAAGTTT GTACAAAAAAGCAGGCTTAGAAAACCTGTACTTCCAG GGTCCCGCTACCACTAATCAACAAATC-3' and R 5'-GGGGAC CACTTTGTACAAGAAAGCTGGGTCTTATTAAAAATAAA GATCGAACTGTGCCGTT-3'. This construction was cloned into pDONR 201 vector according to BP reaction, and then transferred into pETG-20A vector (EMBL) according to LR reaction with Ampicilin selection. The obtained construction was N-terminally tagged by 6 x His and fused with thioredoxine. It contained the sequence of cleavage by the protease TEV (underlined in primer sequence). The sequence of plasmid that contains gene CBU_0937 was confirmed by sequencing.

Purification of recombinant proteins. Expression and purification of Com1 and CBU_0937 was performed as previously described (Berrow *et al.*, 2006; Vincentelli *et al.*, 2005) and briefly exposed here. To overcome the problems of protein solubility of proteins Com1 and CBU_0937, the predicted N-peptide signal (http://bp.nuap.nagoya-u.ac.jp/sosui) was removed from the protein sequence Com1 (MKNRLTALFLAGTLTAGVAIAAPSQF) and CBU_0937 (MTSKLVISALGLCVSGALSTTLAST), respectively. The plasmids containing gene *Com1* (CBU_1910) and gene *CBU_0937* were transformed into *Escherichia coli* strains Rosetta (DE3) pLysS (Novagen) and the bacteria were grown in the auto-induction medium ZYP5052, then collected and resuspended in lysis buffer, frozen -80°C for at least 1hr and treated by DNase I/$MgSO_4$ as previously described (Studier *et al.*, 2005, Vincentelli *et al.*, 2005). The lysed cells were centrifuged to separate the soluble (supernatant) from insoluble fraction (pellet). For Com1, the supernatant (soluble) was used for subsequent steps of purification;

CBU_0937 was searched in the pellet (insoluble). The proteins were purified by affinity chromatography based on the affinity of the 6xHis tag with Ni ions according to the standard procedures (Vincentelli *et al.*, 2005). The protein Com1 was eluted under native conditions (50 mmol/l Tris, pH 8.0, 300 mmol/l NaCl, 250 mmol/l imidazole) and CBU_0937 was eluted under denaturing conditions (50 mmol/l Tris, pH 8.0, 300 mmol/l NaCl, 250 mmol/l imidazole, 6 mol/l urea). The fractions containing Com1 and CBU_0937 proteins were pooled and stored in 50% glycerol at -20°C until use for ELISA. The identity of protein Com1 and CBU_0937 was confirmed by mass spectrometry.

ELISA. Modified ELISA was performed as previously described (Nde *et al.*, 2002; Lau *et al.*, 2005). Briefly, 96 well plates were coated overnight at +4°C with recombinant protein Com1 and CBU_0937 (5 μg/ml, 100 μl per well) diluted in carbonate-bicarbonate buffer (15 mmol/l Na_2CO_3, 35 mmol/l $NaHCO_3$, pH 9.6). Alkaline phosphatase-conjugated goat anti-human IgG (Sigma) was used as a secondary antibody. Color development was assessed at A_{405} and data analyzed by GraphPad Prism. IF-positive serum from the patient with active *C. b.* infection and IF-negative serum from the healthy donor served as the positive and negative control, respectively. Each serum sample was tested in triplicate. Cut-off was determined as a mean of negative IF samples plus 1.5 x of standard deviation (Beare *et al.*, 2008). Any sample exhibiting an absorbance above the cut-off value (Com1; 0.4423) (CBU_0937; 0.33485) was considered as positive.

Results

Identification of the immunoreactive C.b. proteins

We have successfully resolved 250 protein spots of *C. b.* strain Nine Mile within the pH range from 3 to 10 (7 cm gels). *C. b.* proteins were probed with four MAbs 2-13-1, 3-3-4, 8-2-2, and 1-4-H, and immune rabbit serum (Fig. 1b, c, d, e, and f, respectively). While MAbs 2-13-1 and 3-3-4 recognized the same proteins, antigenic sites revealed by MAb 8-2-2 were different, and with the MAb 1-4-H the reaction was negative. The immunoreactive proteins detected after silver staining identified by MS (Fig. 1a) belonged mainly to the three classes of proteins listed in Table 2.

The antigen reacting with MAbs 2-13-1 and 3-3-4 was identified as a conserved hypothetical protein of unknown function (CBU_0937) and another antigen reactive with MAb 8-2-2 was identified as outer membrane protein (CBU_1910, Com1). The translation elongation factor Tu (tuf) (CBU_0236) also reacted with this MAb. We have carefully checked and manually edited all mismatches found on the immunoblots using a rigorous strategy recommended by Dowsey *et al.* (2003, 2004).

Cloning, expression, and purification of recombinant proteins Com1 and CBU_0937

The gene size was determined after amplification by PCR. The Com1 gene was about 759 bp and CBU_0937 gene was 1,320 bp (Fig. 2a, c). The results of the PCR, sequencing of construction plasmid, as well as the restriction fragment length polymorphism reaction showed that the *C. b.* gene Com1 was cloned into the expression plasmid pIVEX 2.3d. Recombinant 6 x His tagged proteins Com 1 and CBU_0937 reacted with MAb anti-RGS-his.

The recombinant protein Com1 exhibited M_r around 27 K (Fig. 2b) that is close to the predicted M_r of 27.8 K by the genome. The recombinant protein CBU_0937 exhibited M_r around 65 K (Fig. 2d) that is close to the theoretical M_r 63.6 K predicted for this construction containing thioredoxine.

The proteins were purified by Nickel affinity chromatography, analyzed by SDS-PAGE, and displayed the bands at 27 K and 65 K, respectively (Fig. 2b, d).

Screening of sera of Q fever patients by ELISA using recombinant proteins Com1 and CBU_0937

In total 48 human sera were tested in recombinant ELISA for the presence of IgG antibodies (Fig. 3). We tested 34 patients with the active *C. b.* infection (18 patients with Q fever IE and 16 patients with Q fever acute), and 14 control sera from the healthy donors (Table 2). The sera displaying A_{405} higher than determined cut-off

Table 2. Identification of C. b. proteins discriminated by MAbs

Reactive MAb	*C. b.* protein	Locus	Locus tag	NCBI (Acc. No.)	M_r	pI
2-13-1	Putative protein	–	CBU_0937	gi|29541524	51,6 K	8.99
3-3-4	CBU_0937					
8-2-2 (weakly)						
8-2-2	Outer membrane Com1 protein†	Com1	CBU_1910	gi|29542467	27,7 K	9.23
8-2-2	Elongation factor Tu	tuf-2	CBU_0236	gi|29540850	43 K	5.32
8-2-2 (unspecific)	Serum albumin	BSA	BSA	gi|1351907	71,2 K	5.82

pI = theoretical isoelectric point; † = potentially transmembrane protein.

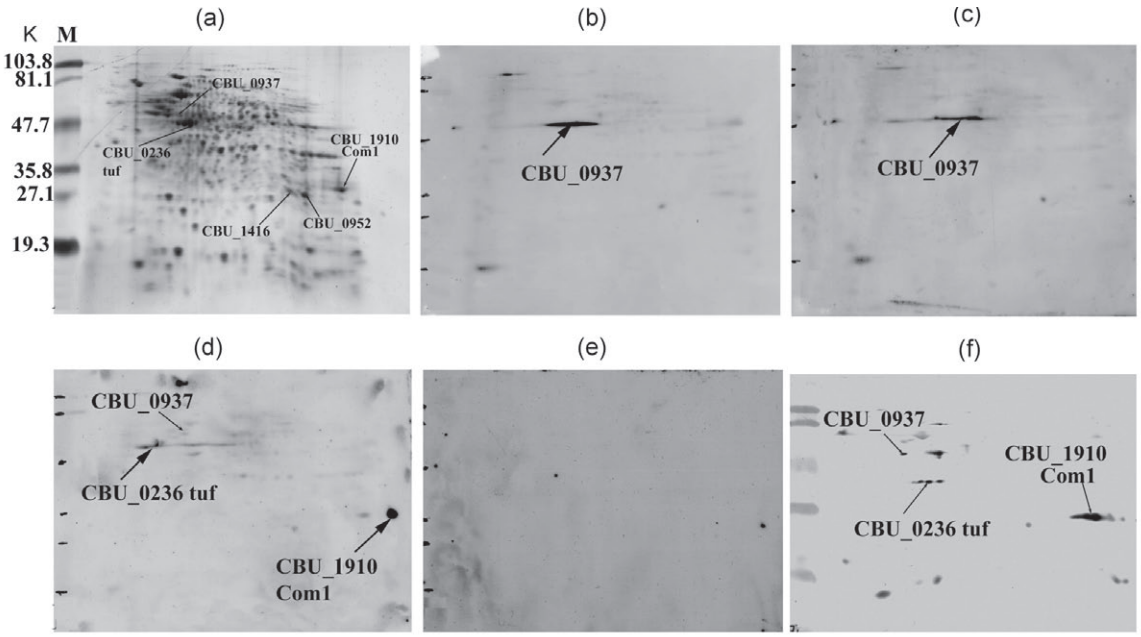

Fig. 1

2-D immunoblot analysis of *C. b.* proteins using MAbs and a polyclonal antiserum

Silver-stained proteins (a) with protein size markers (M). Immunoblots probed with 1:100 dilutions of MAbs 2-13-1 (b), 3-3-4 (c), and 8-2-2 (d), 1:2 dilution of MAb 1-4-H (e), and polyclonal antiserum (f).

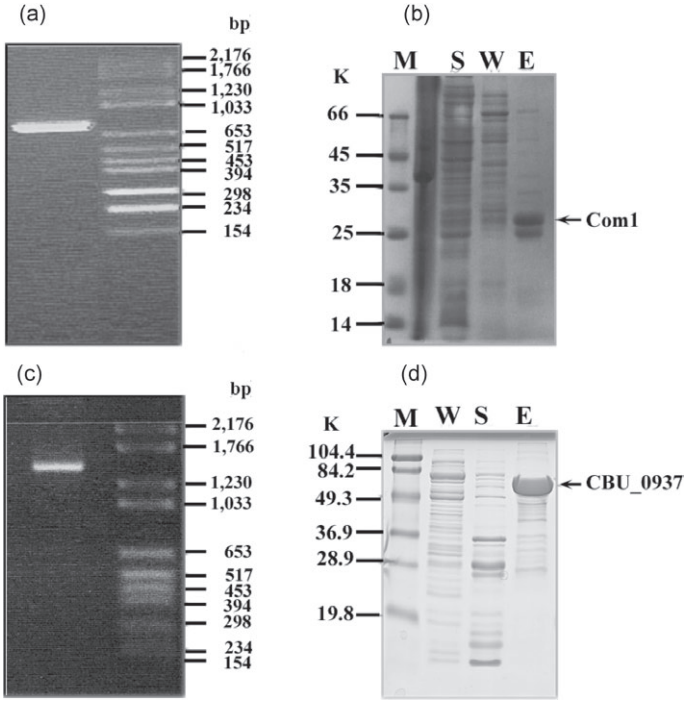

Fig. 2

PCR-amplification of Com1 and CBU_0937 genes and purification of respective recombinant proteins

SDS-PAGE of PCR-amplified Com1 (a) and CBU_0937 (c) genes. SDS-PAGE of purification fractions of Com1 (b) and CBU_0937 (d) proteins. Protein size markers (M), supernatant (S), wash (W), and eluate (E).

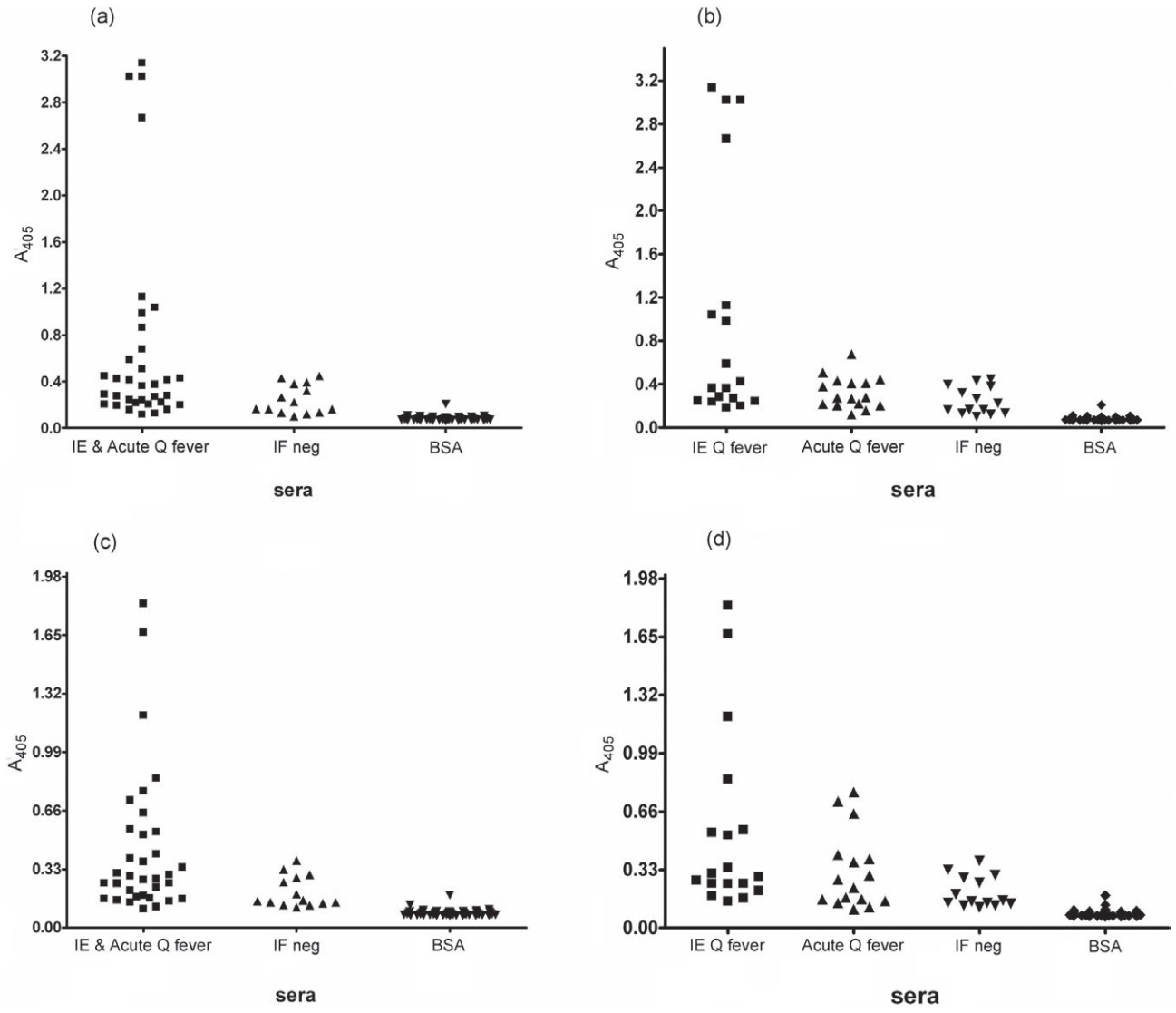

Fig. 3

Screening of Q fever sera by ELISA using recombinant proteins Com1 (a, b) and CBU_0937 (c, d)

Total sera (a, c) and acute Q fever and IE sera (b, d).

value for Com1 were mostly from patients with Q fever IE (10/18) (Table 3). Only 6 patients with acute Q fever were positive in ELISA.

When considering all Q fever patients together, the sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), negative likelihood ratio (NLR), as previously defined were 47 %, 71.4 %, 80%, 35.7%, 1.62, and 0.74, respectively, (Stein *et al.*, 1994). The results of ELISA obtained for CBU_0937 with all sera were in the same range as consideration of both groups separately (Se 38%, Sp 93%, PPV 93%, NPV 38%, PRL 5.27, NRL 0.67).

Discussion

The availability of *C. b.* genome and the development of proteomic technologies have opened the framework for proteins identification useful in clinical applications. *C. b.* genome annotation contains a high portion of hypothetical proteins that needs to be tested for the biological competence (Seshadri *et al.*, 2003). Recently, several proteomic studies have been reported using polyclonal sera, but none has submitted utilization of MAbs (Škultéty *et al.*, 2007; Coleman *et al.*, 2007; Samoilis *et al.*, 2007). Application of MAbs in *C. b.* immunoproteomic studies is a valuable tool to

Table 3. Screening of Q fever sera by ELISA using recombinant proteins Com1 and CBU_0937

Recombinant protein	Sera	Cut-off luvalue	Se (%)	Sp (%)	PPV (%)	NPV (%)	PRL	NRL	References
Com1	Total	–	50	90	–	–	–	–	(Beare *et al*., 2008)
Com1	Total	–	100	100	–	–	–	–	(Zhang *et al*., 1998)
Com1	Total	0.43	47	71	80	36	1.6	0.74	This study
Com1	Q fever IE	0.43	55	71	71	55.5	1.9	0.63	This study
Com1	Acute Q fever	0.43	37.5	71	54.5	47	1.3	0.875	This study
CBU_0937	Total	0.33	38	93	93	38	5.3	0.67	This study
CBU_0937	Q fever IE	0.33	39	93	87.5	54	5.4	0.66	This study
CBU_0937	Acute Q fever	0.33	37.5	93	86	56.5	5.2	0.67	This study

NPV = negative predictive value; NRL = negative likelihood ratio; PPV = predictive positive value; PRL = positive likelihood ratio; Se = sensitivity; Sp = specificity.

depict the proteins of choice. We have exercised two MAbs selected recombinant proteins for precise confirmation of acute versus chronic stages of the Q fever disease, especially stressing Q fever IE cases.

The protein Com1 is known to be preferentially exposed on the surface of *C. b.* (Hendrix *et al*., 1993) and is continuously applied for seroimmunological screenings. The Com 1 gene coding for this protein was applied as a genetic marker to distinguish acute and chronic isolates (Zhang *et al.*, 1998). The results obtained in our study skewed with those of the recent study by Beare *et al*. (2008). The values of specificity and sensitivity obtained with Com1 protein were in the range of our results, 90% and 50% respectively. However, not all chronic Q fever cases may evolve into IE. Consequently, screening of IE patients sera with cloned *C. b.* proteins was a main interest for us. We were able to show that in ELISA the values of these sera were slightly enhanced in comparison to those detected by all Q fever patients or when measured with acute Q fever patients only (Table 3). Our results indicated that ELISA using Com1 would be better applicable for the diagnosis of Q fever IE than for Q fever acute cases.

In our study, protein CBU_0937 was described as an antigenic target for diagnosis of Q fever for the first time. The results were more promising, but considering all patients together, the low value of sensitivity was offset by high range of specificity (Se 38%, Sp 92.8%). When comparing the results within the groups, Q fever IE versus acute Q fever to all patients, we did not see any evolution in the test-operating parameters. The PRL was 5.27 < 10 indicated only minor contribution of CBU_0937 based ELISA in the clinical serodiagnosis. The value of specificity and PPV skewed towards those used in clinical applications, but lacked sensitivity.

Taken together, the results of ELISA based on the recombinant proteins Com1 and CBU_0937 showed that they were both usable and comparable to the commercial kit available for bacterial antigens detection (Se = 75%, Sp = 95%) (Petrov *et al.,* 1990; Shah *et al*., 2006).

Application of immunoproteomic results to the medical practice depends on a progress to refine MAbs-based screening technologies. We are aware of the fact that a larger set of MAbs may markedly enhance the credibility of proposed technique for the future development of diagnostic kits.

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