

RESEARCH ARTICLE

Genome-wide profiling of humoral immune response to *Coxiella burnetii* infection by protein microarray

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Comprehensive evaluation of the humoral immune response to *Coxiella burnetii* may identify highly needed diagnostic antigens and potential subunit vaccine candidates. Here we report the construction of a protein microarray containing 1901 *C. burnetii* ORFs (84% of the entire proteome). This array was probed with Q-fever patient sera and naïve controls in order to discover *C. burnetii*-specific seroreactive antigens. Among the 21 seroreactive antigens identified, 13 were significantly more reactive in Q-fever cases than naïve controls. The remaining eight antigens were cross-reactive in both *C. burnetii* infected and naïve patient sera. An additional 64 antigens displayed variable seroreactivity in Q-fever patients, and underscore the diversity of the humoral immune response to *C. burnetii*. Nine of the differentially reactive antigens were validated on an alternative immunostrip platform, demonstrating proof-of-concept development of a consistent, safe, and inexpensive diagnostic assay alternative. Furthermore, we report here the identification of several new diagnostic antigens and potential subunit vaccine candidates for the highly infectious category B alphaproteobacteria, *C. burnetii*.

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1 Introduction

Coxiella burnetii is a gram-negative, obligate intracellular bacteria, and the etiological agent of Q fever [1]. Distribution of *C. burnetii* is global, with infections occurring in a variety of mammals, birds, reptiles, fish, and ticks [2]. Sheep, goats, and other livestock are the primary reservoirs of *C. burnetii*. However, infection of domesticated pets has also been noted. *Coxiella* infection of these animals is usually

asymptomatic, but can lead to abortions in goats and sheep. During birthing, large numbers of bacteria are shed within the amniotic fluids and placenta. The bacteria has a high degree of extracellular stability and is highly resistant to heat, drying, and many disinfectants. The organism is readily transmitted through birth fluids, ingestion of unpasteurized dairy products, excreta of infected herd animals, and airborne barnyard dust contaminated by dried placental material. As such, farm animals and pets are the main reservoirs of infection for humans [3]. Humans are highly susceptible to infection, as *C. burnetii* is considered one of the most infectious bacteria, with an ID₅₀ of 1. For these reasons, *C. burnetii* is considered a potential bioweapon and is classified as a category B agent by the US Centers for Diseases Control and Prevention.

In humans, Q fever is a self-limiting but debilitating influenza-like illness. *C. burnetii* infection is considered to be under-reported because diagnosis often remains difficult

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Abbreviations: HA, hemagglutinin; HIS, histidine; IVTT, *in vitro* transcription-translation; TAP, transcriptionally active PCR; TTBS, Tris buffer containing 0.05% v/v (pH 8.0) Tween-20

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and the infection may be asymptomatic in half of infected patients. Symptoms of acute Q fever are often broad and include prolonged high fever, severe headache, confusion, vomiting, diarrhea, and malaise resulting in a 1–2% mortality rate. Chronic Q fever develops in 0.2% of infections, and can be fatal if left untreated. Current advances in treatment (including the combination of doxycycline/hydroxychloroquine) have successfully limited the mortality rate for Chronic Q fever to less than 1% [1, 4, 5]. Chronic infections may cause life-threatening endocarditis but may not show apparent symptoms, leading to under-reporting. The occurrence of both acute and chronic disease in humans has been linked to predisposing host factors [6, 7].

Current diagnosis of Q fever is based on several methods of detection including: indirect immunoperoxidase assay [8], ELISA [9–11], monoclonal antibodies for paraffin-embedded tissues [12], PCR-based assays [13], microagglutination [14, 15], complement fixation test, and indirect immunofluorescence assay. The latter two are the only commercially available diagnostic assays and require purified phase I and phase II organisms as antigens. Since production of whole organisms is difficult and hazardous, there is an imperative need for alternative serodiagnostic reagents, including recombinant proteins. Typically, recombinant protein-based diagnostic assays have less inconsistency than whole-cell-based assays, and increased specificity. However, the serodiagnostic antigens of *C. burnetii* have not been well characterized on a comprehensive proteomic level, and warrant thorough investigation.

High-density proteome microarrays offer an effective means for determining the complete antigen-specific antibody response to infection on a genome-wide scale [16–26]. Unlike 2-DE, protein microarrays can be fabricated in large numbers so that individual patient specimens can be conveniently and quantitatively interrogated, enabling a more complete understanding of the extent and diversity of the host response to infection on a patient-specific and antigen-specific basis, across the complete proteome. Vaccine and serodiagnostic antigens against several infectious agents have been discovered in this way [16, 20, 22, 24]. For these reasons a first generation *C. burnetii* proteome microarray was fabricated using transcriptionally active PCR (TAP) fragments, probed with a small collection of Q-fever patient specimens ($n = 5$), and a set of seroreactive antigens were identified [18]. Beare *et al.* [18] then cloned the identified antigens into expression plasmids and directly compared microarrays containing proteins expressed in *in vitro* transcription-translation (IVTT) reactions from plasmids to TAP fragments. Beare *et al.* [18] concluded increased reactivity in plasmid-driven IVTT expressed proteins. In this report we have cloned the complete proteome into expression plasmids and fabricated a comprehensive *C. burnetii* proteome array produced entirely (1901 ORFs) from the plasmid based expression system and probed with a large collection of patient ($n = 40$) and control sera ($n = 20$). Furthermore, we identified a set of differentially reactive and cross-reactive antigens, and vali-

dated the diagnostic potential of these antigens using an alternative Western-blot style immunostrips platform (also called line blots).

2 Materials and methods

2.1 PCR amplification of linear acceptor vector

ORFs were cloned into pXT7 vector using a high-throughput PCR cloning method previously described [21]. pXT7 plasmid (3.2 kb, KanR) encoding an N-terminal 10 × histidine (HIS) tag and a C-terminal hemagglutinin (HA) tag was linearized with *Bam*HI (0.1 µg/µL DNA, 0.1 mg/mL BSA, 0.2 U/µL *Bam*HI, Invitrogen) overnight at 37°C. The digest was purified using PCR purification kit (Qiagen, Valencia, CA, USA), quantified using a NanoDrop (Thermo Scientific), and verified by agarose gel electrophoresis. PCR was used to generate the linear acceptor vector in 50 µL PCRs with 0.5 µM of each primer (CTACCCATACGATGTTCCGGATTAC and CTCGACATATGCTTGTCTCGTCGTCG). PCR was performed using 0.02 U/µL AccuPrime *Taq* DNA polymerase (Invitrogen), 0.8 mM dNTPs, and 1 ng pXT7 diluted in AccuPrime Buffer II using the following conditions: 95°C for 5 min 30 cycles of 95°C for 0.5 min, 50°C for 0.5 min, 72°C for 3.5 min, and a final extension of 72°C for 10 min.

2.2 ORF cloning

All *C. burnetii* (AE016828) ORFs larger than 150 bp were attempted to be cloned using 20 bp ORF sequence-specific PCR primers to the 5' and 3' ends. A unique 20 bp homologous recombination “adapter” sequence was included on the end of the 5' and 3' ORF specific primers (ACGA-CAAGCATATGCTCGAG and TCCGGAACATCGT-ATGGGTA, respectively). The adapter sequences, which become incorporated into the termini flanking the amplified gene, are homologous to the cloning sites of the linearized T7 expression vector pXT7 and allow for high-throughput cloning without the need for restriction or ligase enzymes. PCRs were prepared using 0.02 U/µL AccuPrime *Taq* DNA polymerase (Invitrogen), 0.8 mM dNTPs, diluted in Buffer II, with 2.5 ng of *C. burnetii* template with the following conditions: 95°C for 2 min, 30 cycles of 95°C for 0.33 min, 55°C for 0.25 min, 50°C for 0.25 min, 68°C for 3 min, and a final extension of 68°C for 10 min. All *C. burnetii* ORF-PCRs were confirmed by gel electrophoresis for correct insert size prior to cloning into pXT7.

2.3 High-throughput recombination cloning

Linearized pXT7 was diluted to 10 ng/µL, mixed with 1 µL of *C. burnetii* ORF PCR mixture at a volume ratio of 4:1, and incubated on ice for 2 min, followed by addition of 10 µL of

competent DH5 α cells. Reactions were mixed, incubated on ice for 30 min, heat shocked at 42°C for 1 min, and chilled on ice for 2 min. Aliquots of 250 μ L of SOC media were added and cells were incubated for 1 h at 37°C. The whole reaction mixtures were added to 1.5 mL of LB medium with 50 μ g/mL of kanamycin, and incubated overnight at 37°C with shaking. Plasmids were isolated using QIAprep 96 Turbo kits (Qiagen) without colony selection. Minipreps of all 2078 attempted clones were analyzed by agarose gel electrophoresis to confirm insert size. Ninety-five percent of all clones were confirmed for insert size by PCR using ORF sequence-specific primers. An additional 25% of all clones were selected at random and sequenced in both directions. Sequences were analyzed for fidelity, orientation, and for mutation in the overlapping region of the homologous recombination sites.

2.4 Protein microarray chip printing

The expressions of cloned ORFs were carried out for 5 h in IVTT reactions (RTS 100 kits from Roche) according to the manufacturer's instructions. Protein microarrays were printed onto nitrocellulose coated glass FAST slides (Whatman) using an Omni Grid 100 microarray printer (Genomic Solutions). Aliquots of 3.3 μ L of 0.2% Tween-20 were mixed with 10 μ L of IVTT and transferred to 384-well plates. Plates were centrifuged at 1600 $\times g$ to pellet any precipitate and remove air bubbles prior to printing. Supernatants were printed immediately without purification, and all ORFs were spotted in duplicate. Data values reported herein represent average of the pair, unless otherwise mentioned. In addition each chip was printed with control spots consisting of IVTT reaction without plasmid, purified Vaccinia immune globulin, and purified EBNA1 protein. Vaccinia immune globulin and EBNA1 were obtained from ADi as a gift and printed 16 times in serial dilution on each microarray and can be seen on the representation microarray images in Fig. 1. Protein expression was confirmed by using monoclonal anti-poly-HIS (clone HIS-1, Sigma) and anti-HA (clone 3F10, Roche).

2.5 Microarray probing

Thirty-two Q-fever patient sera from Australia (Dr. John Stenos, Australian Rickettsial Reference Laboratory, Geelong Hospital, Geelong VIC 3220) were acquired through approved exempt protocol NMRC.2008.0006. Samples were collected with IRB approval during outbreaks. All specimens are stored without identifiers and the identity of the individual patients cannot be ascertained. Sera were obtained from eight US soldiers whose clinical presentations were characterized by rapid onset of fever and chills. Diagnoses were made based on the individual's medical history and physical examination findings, as previously

reported [27]. Together, the serologies of these 40 acute Q-fever cases were compared with 20 healthy USA domestic naïve control sera. Brucella human sera were obtained from patients enrolled in a prospective clinical study of brucellosis in Lima, Peru and were approved by the Comité de Ética of Universidad Peruana Cayetano Heredia, Lima, Peru and the Comité de Ética of Asociación Benéfica PRISMA, Lima.

Sera were diluted to 1/200 in Protein Array Blocking Buffer (Whatman) containing *Escherichia coli* DH5 α lysate (McLab) at a final concentration of 30% v/v, and incubated at room temperature for 30 min with constant mixing. The protein microarrays were rehydrated in Blocking Buffer for 30 min and probed with the pre-absorbed sera overnight at 4°C with constant agitation. The slides are then washed five times in Tris buffer containing 0.05% v/v (pH 8.0) Tween-20 (TTBS), and incubated in biotin-conjugated goat anti-human immunoglobulin (anti-IgGfcy, Jackson Immuno Research) diluted 1/200 in blocking buffer. After washing the slides three times in TTBS, bound antibodies were detected by incubation with streptavidin-conjugated SureLight[®] P-3 (Columbia Biosciences). The slides were then washed three times in TTBS and three times in Tris buffer without Tween-20 followed by a final water wash. The slides were air dried after brief centrifugation and analyzed using a Perkin Elmer ScanArray Express HT microarray scanner.

2.6 Immunostrip assay

Twenty ORFs from sequence-confirmed plasmids were expressed in 5 h IVTT reactions according to the manufacturer's instructions. Proteins were printed on Optitran BA-S 85 0.45 μ m nitrocellulose membrane (Whatman) using BioJet dispenser (BioDot) at 1 μ L/cm, and cut into 3 mm strips. Individual strips were then blocked for 30 min in 10% nonfat dry milk dissolved in TTBS. Prior to immunostrip probing, sera was diluted to 1/250 in 10% nonfat dry milk solution containing *E. coli* lysate at a final concentration of 20% v/v and incubated for 30 min with constant mixing at room temperature. Pre-treated sera were then applied to each strip and incubated overnight at 4°C with gentle mixing. Strips were washed five times in TTBS, and then incubated for 1 h at room temperature in alkaline phosphatase conjugated donkey anti-human immunoglobulin (anti-IgG, Fcy fragment-specific, Jackson Immuno Research), which was diluted to 1/5000 in TTBS. The strips were then washed three times in TTBS, followed by another three washes in tris buffer without Tween-20, and reactive bands were visualized by incubating with 1-step NBT/BCIP developing buffer (Thermo Fisher Scientific) for 2.5 min at room temperature. The enzymatic reaction was stopped by washing the strips with tap water. Strips were air dried and scanned at 2400 dpi (Hewlett-Packard scanner). Images were converted to gray scale format by Photoshop. Unaltered images are shown.

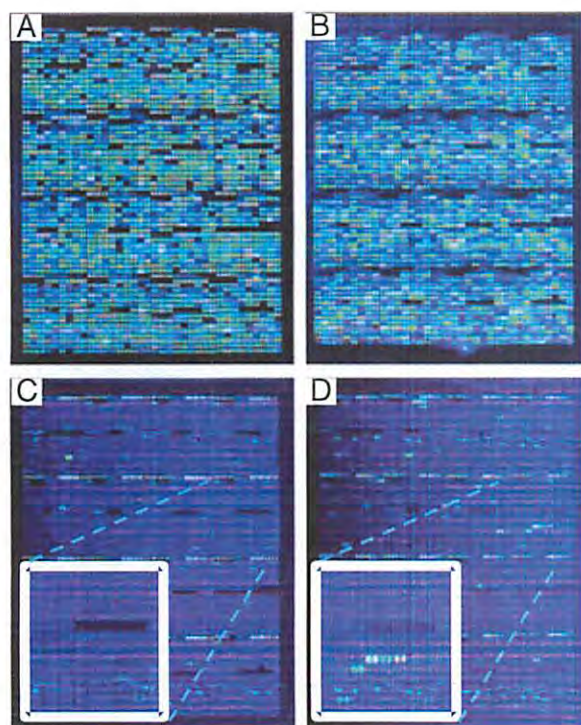


Figure 1. Construction of a *C. burnetii* microarray. Arrays were printed containing 1901 *C. burnetii* ORFs from IVTT reactions. Proteins were printed in duplicates and each array contains positive IgG control spots printed from six serial dilutions of human IgG, six serial dilutions of EBNA1 protein, and six “No DNA” negative control spots. The array was probed with anti-HIS (A) and anti-HA (B) antibody as described in Section 2 to confirm the protein expression and spot reproducibility. Representative protein microarray images of uninfected (C) and infected (D). The arrays were read in a laser confocal scanner and the data normalized as described in Section 2. The signal intensity of each antigen is represented by rainbow palette of blue, green, red, and white by increasing signal intensity. Arrays were probed with 40 Q-fever and 20 control serum samples as described in Section 2.

2.7 Data and statistical analysis

Protein microarrays were scanned and analyzed using a Perkin Elmer ScanArray Express HT microarray scanner. Intensities are quantified using QuantArray Microarray Analysis software. All signal intensities are automatically corrected for spot-specific background. Proteins are considered to be expressed if either tag's signal intensity is greater than the average signal intensity of the IVTT reaction without plasmid, plus 2.5 times the standard deviation. “NoDNA” controls consisting of IVTT reactions without addition of plasmid were averaged and used to subtract background reactivity from the unmanipulated raw data. These results herein are expressed as signal intensity. *p*-Values were calculated using two-tailed Student's *t*-test of unequal variance. Seroreactive antigens with *p*-values less

than 0.05 were considered differentially reactive and seroreactive antigens with *p*-values greater than 0.05 were considered cross-reactive.

Computational prediction of transmembrane domains of the *C. burnetii* proteome utilized the TMHMM v2.0 software [28] found here <http://www.cbs.dtu.dk/services/TMHMM/>, signal peptide prediction used SignalP v3.0 software [29] found here <http://www.cbs.dtu.dk/services/SignalP/>, and cellular location prediction utilized PSORTb v2.0.4 software [30] found here <http://www.psort.org/psortb/>. Enrichment statistical analysis was performed in the R environment, using Fisher Exact test.

3 Results

3.1 Gene amplification and cloning

The proteome of *C. burnetii* strain RSA 493 was cloned using a high-throughput PCR recombination cloning method developed in our laboratory [21]. Custom PCR primers comprising 20 bp of gene-specific sequence with 20 bp of “adapter” sequences are used in PCRs with genomic DNA as template. The adapter sequences were designed to be homologous to the cloning site of the linearized T7 expression vector pXT7, which allowed the PCR products to be cloned by homologous recombination and transformation into *Escherichia coli* DH5 α cells. The *C. burnetii* ORFs were amplified using primers designed to clone all 2077 ORFs in the *C. burnetii* genome larger than 50 amino acids in length. One ORF (CBU0231) was split into two segments based on its length and therefore 2078 cloning reactions were performed. Of the 2078 cloning reactions 1974 were successful (including both segments of CBU0231). All clones were verified for presence of insert by gel electrophoresis and 955 out of those clones were confirmed for insert using ORF-specific primers in PCRs. Twenty-five percent of the cloned ORFs were selected at random and sequenced in both directions to verify target sequence match, orientation, and presence of mutations in the homologous overlapping region during homologous recombination. In >99% of the sequences, the correct insert was verified.

3.2 Construction of a *C. burnetii* protein microarray

C. burnetii ORFs cloned into the pXT7 vector were expressed under the T7 promoter in a 5 h *E. coli* based cell-free IVTT reaction according to manufacturer's instructions. Proteins were printed using an Omni Grid 100 microarray printer (Genomic Solutions) and analyzed for fluorescence on a Perkin Elmer ScanArray Express HT microarray scanner. Proteins were printed in duplicate and evaluated for expression. IVTT expression efficiency was determined by probing against the amino-terminal HIS and carboxy-terminal HA tags for each spot (Fig. 1A and B). Anti-HIS (clone

HIS-1, Sigma-Aldrich, St. Louis, MO, USA) and anti-HA (clone 3F10, Roche) antibodies are conjugated to biotin. Bound antibodies are detected by incubation with streptavidin-conjugated SureLight[®] P-3 (Columbia Biosciences). Intensities are quantified using QuantArray software package. All signal intensities are corrected for spot-specific background. Proteins are considered to be expressed if either the HA or HIS tag signal intensity is greater than the average "No DNA" signal intensity plus 2.5 times the standard deviation, resulting in 96.3% of the *C. burnetii* considered positively expressed. Duplicate printing of protein spots was highly reproducible ($R^2 = 0.986$) and average reactivity for both spots was used in all calculations. In this manner, a protein microarray comprised of 4609 spots was fabricated, consisting of 1901 ORFs of *C. burnetii* strain RSA 493, with positive and negative controls.

3.3 Immune screening

The *C. burnetii* microarray was probed with 40 Q-fever positive sera and 20 healthy naïve samples. Representative microarray images of *C. burnetii* infected and naïve samples are shown in Figs. 1C and D. Signal intensities of duplicate spots were recorded and averaged. The seroreactivity for each antigen was recorded for each patient individually. Antigens were considered seroreactive if the average signal intensity exceeded the average signal intensity of the IVTT reaction without plasmid (no DNA controls) plus 2.5 times the standard deviation. The total IgG antibody response to *C. burnetii* was determined to seroreact with 21 antigens or 1.0% of all of the antigens printed on the array. CBU1910, a 27 kDa outer membrane protein (com1), was the most reactive antigen on average. Twenty-six Q-fever samples showed seroreactivity greater than the "No DNA" average plus 4 standard deviations, and were considered highly reactive. CBU0891, a hypothetical exported membrane associated protein, was the second most reactive antigen,

but was consistently highly seroreactive in most of the individual Q-fever samples ($n = 29$).

3.4 Profile of humoral response in Q-fever patient and naïve controls

Characteristic profiles of antigen reactivity were distinct between Q-fever patients and naïve controls. Seroreactive antigens that are specifically reactive with Q-fever sera but not naïve sera are considered serodiagnostic (p -value ≤ 0.05). Antigens that are not significantly differentially reactive are considered cross-reactive (p -value > 0.05). Cross-reactive antigens may react strongly in Q-fever patients, but are similarly reactive in naïve controls. In contrast, seroreactivity to serodiagnostic antigens is significantly lower in the naïve controls than in *C. burnetii* infected patients. Of the 21 seroreactive antigens, a distinguishing set of 13 serodiagnostic antigens, and eight cross-reactive antigens were identified between infected and naïve groups (Fig. 2). Sera in Fig. 3 were sorted from left to right by increasing average seroreactivity. All seroreactive antigens are grouped as either serodiagnostic or cross-reactive and are sorted in rows with the most reactive antigen to the Q-fever group listed first (CBU1910 and CBU1627, respectively), and the least reactive antigen last (CBU0653 and CBU0436, respectively). The average reactivity of each antigen was compared between the *C. burnetii* infected and naïve samples (Fig. 3). These results showed that many antigens that were not considered seroreactive (on average) were highly reactive for some Q-fever or naïve individual sera (Supporting Information Table 1). While these antigens do not meet our criteria of seroreactivity, they may provide additional insight into the variability of individual humoral immune responses to *C. burnetii*, which may be easily overlooked by other methodologies examining pooled patient samples. The variable immune response we observed is also to be expected in an outbred human population.

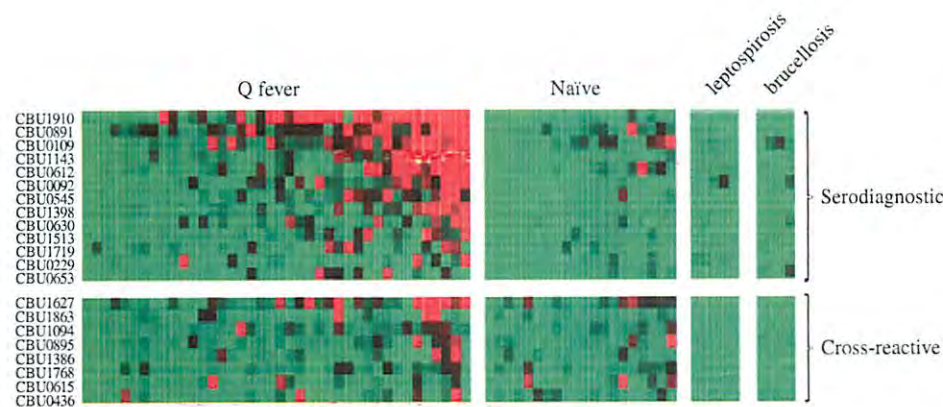


Figure 2. Individual sera are displayed as a heatmap of reactivity. The seroreactive intensity is shown according to the colorized scale with red strongest, black in-between, and green weakest. The antigens are listed in rows and are grouped according to serodiagnostic and cross-reactive. The patient samples are in columns and are sorted left to right by increasing average serodiagnostic antigen intensity.

3.5 Proteomic features of seroreactive antigens

The repertoire of antigen-specific reactivity to *C. burnetii* is shown in Table 1. The data in Table 1 summarize the proteomic features of the 13 serodiagnostic antigens and eight cross-reactive antigens. Eight of the seroreactive antigens contain a signal peptide, and of those, two are predicted to be localized to the outer membrane using SignalP v3.0 and PSORTb v2.0.4 computation software, respectively (both outer membrane proteins were found in the serodiagnostic set). Five serodiagnostic and six cross-reactive antigens are predicted to contain at least one transmembrane domain based on the TMHMM v2.0 computational prediction software [31, 32]. Of the 2272 ORFs in the entire proteome, 1746 do not contain a predicted transmembrane domain, 218 contain a single transmembrane domain, and 308 contain two or more transmembrane domains. The significant enrichment of seroreactive proteins containing predicted transmembrane domains (Fisher Exact p -value = 3.2×10^{-3}) is expected and was observed in a previous protein microarray against *Burkholderia pseudomallei* [16].

3.6 Immunostrip validation and serodiagnosis

To validate the protein microarray seroreactivity and to test the feasibility of transferring these serodiagnostic antigens to an alternative and potentially universal platform, 20 clones were selected, including ten seroreactive antigens (Fig. 4). All 20 clones were single colony purified and sequenced for correct insert in both directions. Immunostrips included a standard curve of human IgG antibody for enzymatic developing consistency. Antigens were expressed in a 5 h IVTT reaction and were printed using a Biojet dispenser. Immunostrips were probed with the entire

collection of sera, and developed as described in Section 2. Reactive bands were visualized after incubation with alkaline phosphatase conjugated anti-human secondary antibody, followed by substrate, and scanned using a desktop scanner at 2400 dpi (Hewlett-Packard scanner). Images were converted to grayscale format and unaltered images of representative immunostrips are shown in Fig. 4. In Fig. 4, clear and distinct reactive bands can be visualized in *C. burnetii* infected sera compared with naïve samples. Ten antigens that did not show seroreactivity by protein microarray were printed as negative controls and did not result in detectable signal on the immunostrip, as expected. Further assessment of the diagnostic accuracy with the immunostrip platform (and existing commercial diagnostic assays) will be made using a blinded and appropriate collection of sera samples.

4 Discussion

In this study we have constructed a *C. burnetii* protein microarray to interrogate the humoral immune response to Q fever. The proteome array was probed in high-throughput fashion against 84% of the *C. burnetii* proteome in order to profile the human humoral immune response to infection. In this manner we were able to define the seroreactive response to Q-fever infection, including the identification of 13 serodiagnostic antigens. Nine of these serodiagnostic antigens were tested in a proof-of-principle diagnostic assay, and may have potential in further development of *C. burnetii* diagnostic assays or as vaccine candidates. Use of immunostrips as an alternative platform validated the seroreactivity we found in the microarrays and may provide an inexpensive and simple alternative to current diagnostics. Further investigation of this diagnostic platform and others (e.g. ELISA) will need to be carried out using a traditional

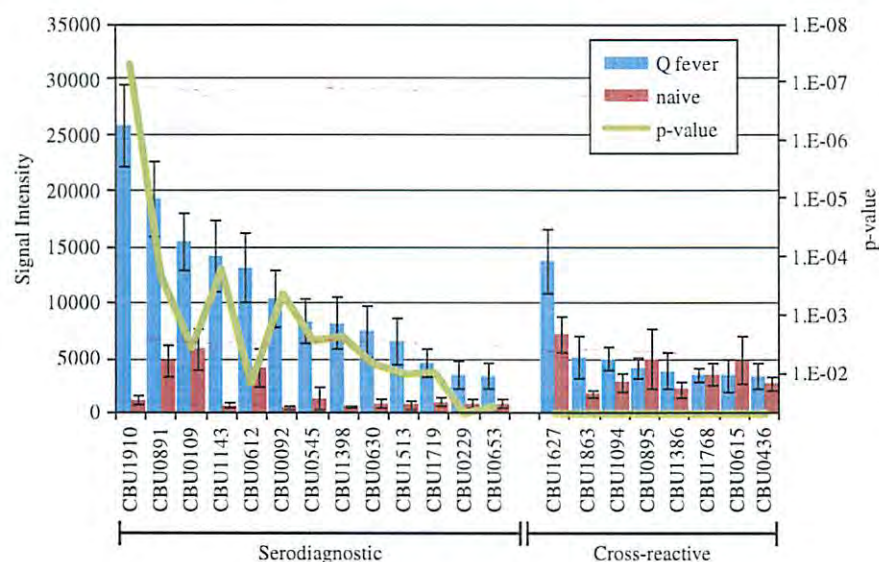


Figure 3. Serodiagnostic and cross-reactive antigen discovery of Q-fever patients. The mean sera reactivity of the 13 antigens was compared between Q-fever infected and US naïve groups. Antigens with a p -value < 0.05 are organized to the left and cross-reactive antigens to the right.

Table 1. Table of all *Coxiella burnetii* seroreactive antigens identified by protein microarray

LocusTag	Gene symbol	Product description	Predicted subcellular location	Transmembrane domains	Signal peptide	Average infected	Average naïve	p-Value	Beare <i>et al.</i> identified
CBU1910	com1	27kDa outer membrane protein	Unknown	1	+	25 816	1188	5.E–08	1
CBU0891		Hypothetical protein, conserved	Unknown	2	+	19 264	4844	2.E–04	
CBU0109		Lipoprotein	Unknown	0	+	15 517	5872	4.E–03	
CBU1143	yajC	Preprotein translocase, YajC subunit	Unknown	1	+	14 235	759	2.E–04	
CBU0612	ompH	Outer membrane protein	Outer membrane	1	+	13 159	4178	1.E–02	2
CBU0092	ygbF	tol-pal system protein YbgF	Unknown	0	+	10 437	537	4.E–04	
CBU0545	lemA	LemA protein	Unknown	1	–	8402	1388	3.E–03	5
CBU1398	sucB	2-Oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase	Cytoplasmic	0	–	8193	633	2.E–03	
CBU0630	mip	Outer membrane protein MIP precursor	Outer membrane	0	+	7481	917	7.E–03	1
CBU1513		Short chain dehydrogenase	Cytoplasmic	0	+	6576	788	1.E–02	
CBU1719	groES	Chaperonin protein Cpn10	Cytoplasmic	0	–	4720	1040	9.E–03	2
CBU0229	rpIL	Ribosomal protein L7/L12	Unknown	0	–	3570	904	5.E–02	
CBU0653		Hypothetical protein, conserved	Unknown	0	–	3473	926	4.E–02	3
CBU1627	lcmE	lcmE protein	Unknown	1	+	13 708	7228	5.E–02	
CBU1863		Hypothetical protein, conserved	Cytoplasmic	4	–	5168	1765	8.E–02	4
CBU1094		Efflux transporter, RND family, MFP subunit	membrane	1	+	5016	2895	1.E–01	
CBU0895		Hypothetical protein	Cytoplasmic	1	–	4186	4981	8.E–01	5
CBU1386	rpsB	Ribosomal protein S2	Unknown	0	–	3886	2207	4.E–01	
CBU1768		Hypothetical protein	Unknown	2	+	3544	3609	1.E+00	6
CBU0615	lpxA	Acyl-[acyl-carrier-protein]–UDP-N-acetylglucosamine O-acyltransferase	Cytoplasmic	0	–	3514	4855	6.E–01	
CBU0436		t-Snare protein, family	Unknown	2	–	3475	2762	6.E–01	7

The horizontal line separates differentially reactive antigens from cross-reactive antigens. Numbers listed under “Beare *et al.*” indicate the seroreactivity rank for each antigen previously identified by Beare *et al.* [18].

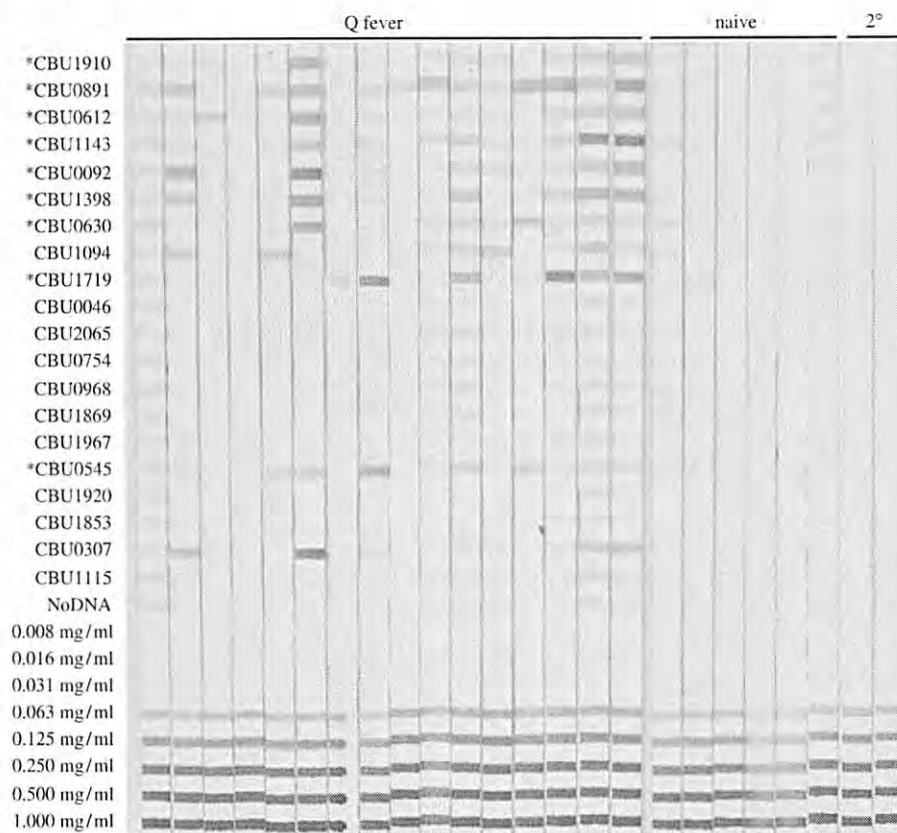


Figure 4. Serodiagnostic antigens were printed onto nitrocellulose paper in adjacent stripes using a BioDot jet dispenser. Antigens that were discovered to be serodiagnostic by protein microarray are indicated by an asterisk. Human IgG was printed on the bottom in serial dilution as a control. Image shows representative strips for infected and naïve human sera samples as well as two strips probed in parallel with secondary antibody only.

blinded study with an appropriate collection of samples to determine sensitivity and specificity.

We have previously reported the seroreactivity of five of the 21 seroreactive proteins using a TAP based protein microarray [18]. The TAP system utilizes a T7 promoter containing PCR product as templates for IVTT reaction. Beare *et al.* identified 44 seroreactive ORFs using 5 individual sera samples. The 44 reactive ORFs discovered using the TAP-based microarray were then compared with the same ORFs cloned into an expression plasmid driven IVTT reaction. Beare *et al.* concluded that protein expression is more efficient in plasmid-driven IVTT than from TAP template-driven reactions, and that detection of seroreactivity was significantly more sensitive when using the plasmid-based IVTT reactions [18]. Because of this earlier work, we developed an expression plasmid-based microarray of all *C. burnetii* ORFs larger than 50aa to comprehensively interrogate the humoral immune response to Q fever. We expected that using the plasmid-based microarray we would have higher sensitivity in detecting antibody response than the TAP-based microarray. As expected, we found that only highly reactive antigens were able to be previously identified. These five antigens (CBU0891, CBU1143, CBU0612, CBU0545, and CBU1398) were the five most reactive antigens using the TAP system. These results support the conclusions by Beare *et al.*, which found that the sensitivity

for seroreactivity of the TAP-based system may indeed be lower than the plasmid-based system. In addition to the antigens discovered by Beare *et al.*, we identify an additional eight serodiagnostic proteins along with all eight cross-reactive proteins.

CBU1910 (Com1), an outer membrane protein, was the most seroreactive antigen, as well as the most significant differentially reactive antigen (p -value = 5×10^{-8}). This antigen has been reported to be targeted by the early humoral immune response in vaccinated cattle [33], and in acutely infected guinea pigs of the Nine Mile strain in phase I [34]. ELISA-based assays using CBU1910 were able to distinguish vaccinated cattle from those naturally exposed [33]. Moreover, CBU1910 vaccinated humanized mice (HLA-DR4 transgenic) have recently been shown to induce a strong γ interferon recall response in purified CD4⁺ T cells [35], consistent with the link between T-helper-cell-mediated antibody response. As previously reported, this antigen was not discovered by the TAP system, due to poor expression from the TAP IVTT reaction system [18]. Nonetheless, Beare *et al.* found that IVTT expressed CBU1910 utilized in a diagnostic ELISA assay had higher specificity than *C. burnetii* cell extract (specificity/sensitivity of 90.0/50.0 compared with 87.5/85.0, respectively) [18].

CBU0891, a hypothetical protein, was the second most reactive protein in our assay. It was the most reactive protein

identified by the TAP system, again showing consistency with previous results. CBU0891 contains a predicted signal peptide by SingalP, but has unknown predicted cellular localization by PSORTb. Further characterization of this protein may provide insight into the reasons why the human humoral immune response targets this antigen to such a high degree.

CBU1143 (YajC) was the fourth most reactive antigen and was also previously identified as serodiagnostic by the TAP microarray. YajC contains a predicted signal peptide and is localized to the inner membrane. YajC is also found to be involved in Sec-dependent secretion [36] and is both a B-cell and T-cell antigen in *Brucella abortus* [37]. We did not find reactivity to this antigen from *Brucella melitensis* infected human sera (Fig. 3). This is likely due to the low sequence homology (39%) between *C. burnetii* strain 493 and the two *Brucella* species (*B. abortus* strain 2308 and *B. melitensis* strain 16M, which share 100% amino acid identity for YajC).

CBU0612 (OmpH) was identified by both microarrays. It contains a signal peptide, is predicted to be localized to the outer membrane by PSORTb, and is reported to be membrane associated [38]. It is one of two outer-membrane predicted that was found to be serodiagnostic. The other outer-membrane predicted protein is CBU0630 (Mip). A partially purified CBU0630 protein was more efficacious in enhancing clearance of organisms from spleens of infected mice than from other proteins or lipopolysaccharide [39]. CBU0109 (the third most reactive antigen), CBU0092, CBU0630, CBU1513, CBU1719, CBU0229, and CBU0653 were not previously identified by the TAP protein microarray and represent novel serodiagnostic antigens.

Additionally, we discovered eight antigens that were cross-reactive among Q-fever, naïve patient, and other bacteremia patient sera. None of these antigens were previously identified by the TAP protein microarray and are presumably the result of humoral response to similar protein structures derived from other unrelated bacterial infections. Furthermore, 64 *C. burnetii* proteins were significantly highly reactive (greater than four times the standard deviation of NoDNA control) to two or more individual Q-fever patient sera and present a variable distribution of reactivity. Of these 64 proteins, 31 were also seroreactive to one or more naïve sera. While the remaining 33 antigens were only seroreactive in the Q-fever sera collection, they did not serve as diagnostic markers, and may represent unique (or limited) immune responses to infection by *C. burnetii*. We listed all 64 of these antigens in the Supporting Information Table 1. Further examination of these antigens may provide novel insight into pathogenesis and the diversity of immune response.

In this comprehensive investigation of the antibody response to the *C. burnetii* proteome, we have found that the seroreactive repertoire targeted only a small percentage of total bacterial proteome (1.0%). Furthermore, a report by Zhang

et al. indicates that the predominant IgG antibody response to phase I organisms recognized proteins and not lipopolysaccharide [40]. Together, these findings suggest that indeed the immunoreactive response to Q fever is very limited. While inclusion of a collection of 40 Q-fever patient samples allowed us to identify many seroreactive antigens, the total number of seroreactive antigens was relatively small and did not present a single universal common proteomic feature. Only 11 of the 21 seroreactive antigens contained a signal peptide, ten contained transmembrane domains, and four were predicted to have membrane localization. This is in marked contrast with the percentage of predicted signal peptide (9.5%) and transmembrane domain containing proteins (23.2%) in the entire proteome. The bias for signal peptide containing proteins was previously observed in a protein microarray screen of *F. tularensis* [22, 26] and *B. pseudomallei* [16]. While the humoral immune response to infection is not stochastic, *in silico* prediction of the antigenic profile based on sequence data alone is still imperfect. We have found that the majority of proteins containing predictive features are mainly nonreactive, and importantly many seroreactive proteins do not contain predictive proteomic features for seroreactivity. For example, six of the 21 seroreactive antigens (28%) identified here do not contain transmembrane domains or signal peptides, and have unknown or cytoplasmic predicted subcellular location. These molecules are unlikely to be predicted by *in silico* prediction algorithms. The results presented here highlight the necessity for empirical determination of seroreactivity and improved *in silico* prediction algorithms for antigen discovery, vaccine development, and insight into the humoral immune response and antigenicity of bacterial pathogens. Currently, the only vaccine against *C. burnetii* infection is a killed cellular vaccine (Q-Vax), which is licensed in Australia [41] and there is no Food and Drug Administration-approved vaccine for human or animal use in the United States, as well as in most countries. Vaccination causes severe local and occasional systemic reaction in patients sensitized to *C. burnetii* and requires a skin test prior to vaccination [42]. Protection against Q fever is reported to involve both cellular and humoral immunity [43]. Successful demonstration of Q-fever vaccination using recombinant proteins has been reported [44–46]. Development of subunit vaccines and improved diagnostic tests that do not rely on hazardous production of whole-cell bacteria is needed. We believe that the comprehensive evaluation of the humoral immune response to *C. burnetii* reported here may provide additional diagnostic tools and valuable identification for potential subunit vaccine candidates. Comprehensive evaluation of the humoral immune response to Q fever is necessary for novel insight into pathogenesis, as well as the development of subunit vaccines and diagnostics based on recombinant proteins.

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The authors have declared the following conflicts of interest. P.L.F. has patent applications related to protein microarray fabrication and has stock positions with Antigen Discovery, Inc. D.M.M. is an employee with Antigen Discovery, Inc.

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