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Protein array of *Coxiella burnetii* probed with Q fever sera

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Coxiella burnetii is the etiological agent of Q fever. To identify its major seroreactive proteins, a subgenomic protein array was developed. A total of 101 assumed virulence-associated recombinant proteins of *C. burnetii* were probed with sera from mice experimentally infected with *C. burnetii* and sera from Q fever patients. Sixteen proteins were recognized as major seroreactive antigens by the mouse sera. Seven of these 16 proteins reacted positively with at least 45% of Q fever patient sera. Notably, HspB had the highest fluorescence intensity value and positive frequency of all the proteins on the array when probed with both Q fever patient sera and mouse sera. These results suggest that these seven major seroreactive proteins, particularly HspB, are potential serodiagnostic and subunit vaccine antigens of Q fever.

***Coxiella burnetii*, protein array, patient sera, seroreactive antigens, HspB**

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Coxiella burnetii, the etiological agent of coxiellosis or Q fever, is an intracellular, acidophilic, gram-negative bacterium, which replicates within the phagolysosome of the eukaryotic monocytes and macrophages [1,2]. However, it can now be grown axenically in a defined medium [3]. Ease of aerosol dissemination, environmental persistence, and high infectivity [4] make *C. burnetii* a serious threat to humans and as such it has been classified as a category B bioterrorism agent [5]. Q fever is a worldwide disease with acute and chronic stages in humans. Acute Q fever manifests as a flu-like illness with high fever, fatigue, and chills, often accompanied by severe headaches. Serious complications and even death can occur in patients with acute Q fever, particularly in those with meningoencephalitis or myocarditis, and in chronic Q fever patients with endocarditis.

An efficacious and safe vaccine and a specific and sensitive serodiagnostic test are required for prevention and control of coxiellosis. Q-Vax, the Q fever vaccine licensed in Australia, is highly efficacious in prevention of the disease by inducing robust humoral and cell-mediated immune responses to *C. burnetii* [6]. However, severe local and occasionally systemic adverse reactions to this vaccine have been observed, particularly among individuals previously sensitized to *C. burnetii* [7]. The diagnosis of human Q fever is mainly based on clinical presentation and serological responses against whole-cell phase I and phase II antigens of *C. burnetii*. Unfortunately, the complex nature of the whole-cell antigens of *C. burnetii* results in a lack of uniformity in test results [8]. Both microimmunofluorescence serological tests and Q fever vaccines contain intact organisms of *C. burnetii* that contain many antigens.

Investigation of *C. burnetii* antigens by various molecular methods could be a valuable research tool for the development of novel approaches for detecting *C. burnetii* infection in clinical samples. Recently, several immunoproteomic studies have reported the identification of such candidate protein antigens [9]. Moreover, many studies have also confirmed that the protein microarray approach is a feasible,

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comprehensive, and high-throughput analysis tool for the elucidation of humoral immune responses to bacterial antigens, and enables the discovery of potential antigens for diagnosis and vaccine development [10–12].

In this study, using genome sequence analysis of *C. burnetii* (Nine Mile, RSA493) [13], genes encoding proteins implicated in adhesion, invasion, intracellular trafficking, host modulation, detoxification, and other putative virulence-related functions (Table S1) were selected for expression in prokaryotic cells. One hundred and one of the selected genes were expressed successfully as recombinant proteins in *Escherichia coli* cells BL21 and subsequently produced as a microarray. The recombinant proteins on the microarray were systematically screened with mouse and human Q fever sera.

1 Materials and methods

1.1 Organism cultivation and chromosomal DNA isolation

The phase I strains of *C. burnetii* (Nine Mile and Xinqiao) [14] were propagated in chicken embryo yolk-sac as described previously [15]. Chromosomal DNA was extracted directly from the infected yolk-sac membranes using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). The extracted DNA was used as the template for the amplification of *C. burnetii* genes by polymerase chain reaction (PCR).

1.2 Sera of mice infected with *C. burnetii*

Forty BALB/c mice (7-week old males) were intraperitoneally inoculated with *C. burnetii* Xinqiao strain (10^7 organisms per mouse), and 10 mice were randomly chosen and sacrificed on days 7, 14, 21, and 28 post-infection (pi). The sera from each group were pooled. The Beijing Administrative Committee for Laboratory Animals approved the animal usage.

1.3 Sera of patients

Sixty-nine Q fever patient sera from various stages of infection were provided by the Australian Rickettsial Reference Laboratory (Geelong, VIC, Australia). Patients were diagnosed using an in-house *C. burnetii* immunofluorescence assay (IFA). According to the results of IFA and clinical symptoms, the Q fever antibody positive (QAb-positive) sera were classified into 3 types, acute, chronic, and past. The acute sera were further classified into 3 subtypes, early, late, and convalescent. Nine sera from patients diagnosed with early stage acute Q fever were Q fever antibody negative (QAb-negative) in IFA and these were used as negative controls in the protein array. This study was approved by the ethics committee of the Beijing Institute of Microbiology and Epidemiology. The serum samples of patients were collected as part of the routine management of patients and all patient data were anonymized.

1.4 Preparation of assumed virulence-associated recombinant proteins

One hundred and fifty-six open reading frames (ORFs), which were assumed to be associated with bacterial virulence, were selected from the *C. burnetii* Nine Mile strain (RSA493) genome sequence (accession number NC_002971). According to the genome annotation of *C. burnetii* in GenBank, a reductive strategy was employed to remove genes encoding proteins present in non-pathogens and housekeeping genes, whilst retaining genes that code for known or suspected virulence-associated proteins (Table S1). The genes encoding these virulence-associated proteins were amplified with primer pairs designed with Primer 5.0 software (Table S2). Adapter sequences homologous to the cloning sites of pET-32a prokaryotic expression plasmid were added to the primers which allowed cloning of the PCR products into the expression plasmid.

The amplified target gene fragments were purified from agarose gels using a DNA purification kit (Qiagen) and ligated to the pET-32a vector (Novagen, Madison, WI, USA) as per the manufacturer's instructions. Competent cells (*E. coli* BL21) were transformed with the plasmids and screened on agar plates containing ampicillin according to standard procedures [16]. Positive clones were cultured in liquid LB and induced with isopropyl-β-D-thiogalactoside (IPTG; Sigma, Louis, MO, USA) to express the recombinant proteins. Following IPTG induction, bacteria were pelleted by centrifugation and suspended in 10 mmol L^{-1} Tris-HCl buffer (pH 8.0). The bacteria were mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer and boiled for 10 min. The proteins were separated by electrophoresis on 12% (w/v) SDS-polyacrylamide gels (SDS-PAGE) and stained with Coomassie blue.

1.5 Microarray fabricated with recombinant proteins

The recombinant proteins were purified using Ni-NTA resin (Qiagen) according to the manufacturer's protocol. The concentration of the purified proteins was determined using a BCA protein assay reagent kit (Pierce, Rockford, MN, USA) and adjusted to approximately 150 μg mL^{-1} with 250 mmol L^{-1} imidazole [17]. The purified proteins were printed in triplicate spots onto aldehyded glass slides (CEL, Pearland, TX, USA) to create a protein array. The cell lysate of *E. coli* BL21 transformed with pET-32a plasmid was printed as a negative control and mouse immunoglobulin G (IgG) or human IgG was printed as a positive control. The printed slides were incubated at room temperature for at least 1 h and then stored at 4°C. For quality control, the proteins were incubated with the Cy5-conjugated anti-His mouse

IgG on the array. Only the proteins with a signal-to-background ratio of ⩾3.0 were used for further analysis.

1.6 Detection of seroreactive proteins

To minimize background reactivity, all sera were diluted 1:200 in PBS buffer (pH 7.2) containing 2% (w/v) bovine serum albumin (BSA). Non-specific interactions were further reduced by incubating the sera for 2 h with the debris of the IPTG induced cells carrying pET32a. The arrays were blocked with PBS containing 1% (w/v) BSA at room temperature for 60 min, and then incubated with the absorbed sera for 12 h at 4°C. The arrays were washed five times in wash buffer (PBS containing 0.05% (w/v) Tween 20). For detection of reactivity, Cy5-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, UK) diluted 1:500 in PBS containing 1% (w/v) BSA and Cy5-conjugated goat anti-human IgG (Jackson Immunoresearch, West Grove, PA, USA) diluted 1:200 were added to their respective assays and incubated at 37°C for 45 min. After three wash buffer rinses, three PBS rinses and one final water wash, the arrays were air-dried for 30 min and then scanned with a Perkin-Elmer ScanArray Express HT apparatus (PerkinElmer, Covina, CA, USA) at a wavelength of 630 nm and with an RGB format TIFF file output.

1.7 Data analysis

The scanned images were analyzed by the GenePix pro 5.1 software (Axon Instruments, Union, CA, USA). The fluorescence intensity (FI) of each protein was calculated by averaging the FIs of three replicate spots with their background fluorescence subtracted. The FIs obtained from different arrays were normalized based on the FIs of the positive controls and displayed with TreeView software [18]. The significance of differences between samples collected from the groups of mice or patients were determined by an exact Wilcoxon signed rank test using SPSS 16 software (IBM, Armonk, NY, USA) [19].

2 Results

2.1 Development of a protein array assay using *C. burnetii* proteins

One hundred and fifty-six genes encoding proteins implicated in adhesion, invasion, intracellular trafficking, host modulation, detoxification, and other virulence-related functions were selected for the protein array. One hundred and seventy-three pairs of primers were designed to amplify the 156 genes (Table S2). Because of the large size of 17 target genes with sequence lengths greater than 2000 bp, amplicons were divided into two fragments for total gene coverage (Table S2). One hundred and fifty-five of the 173 amplified gene fragments were successfully cloned into the pET-32a plasmids and 104 were efficiently expressed as recombinant proteins in *E. coli* cells. Using nickel-ion affinity chromatography (Ni-NTA), 104 recombinant proteins were purified from the cellular materials of the transformed bacteria. These expressed proteins were printed onto an aldehyde slide by the SpotArray^{TM}24 robot to create a subgenomic protein array. One hundred and one proteins giving a signal-to-background ratio of ⩾3.0 were thought to be acceptable for further analysis.

2.2 Seroreactive proteins identified with mouse sera

The 101 proteins on the array were probed with sera from BALB/c mice that had been experimentally infected with *C. burnetii*. The proteins were considered to be seroreactive if their FIs were six-fold higher than those recorded for normal mouse sera [20]. As a result, 2, 66, 84, and 75 of the 101 proteins were recognized as seroreactive by mouse sera obtained at days 7, 14, 21, and 28 pi, respectively. Among these seroreactive proteins, 15 had stronger FIs than that of *Coxiella* outer membrane protein 1 (Com1) [21], when probed with sera obtained on days 21 and 28 pi (Figure 1).

2.3 Major seroreactive proteins identified with patient sera

The 101 *Coxiella* proteins on the array were probed with Q fever patient sera. The average FI value of the proteins probed with QAb-negative sera was 83 (Table S3). The average FI value of the proteins probed with the sera from patients with early stage acute Q fever was 100 (Table S3), slightly higher than that of the negative control, The average FI value of the proteins probed with the sera from patients with late stage acute Q fever was 212 (Table S3), significantly higher than that probed with early stage acute Q fever patient sera (P<0.05) (Figure 2). All the FI values from the

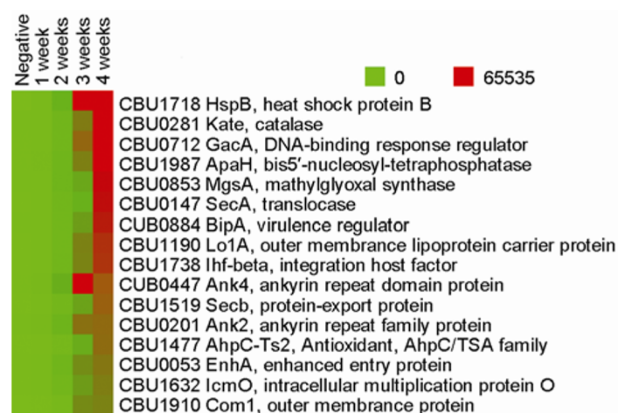

Figure 1 Sixteen proteins on the array were strongly recognized by mouse sera obtained at days 7 (1 week), 14 (2 weeks), 21 (3 weeks), and 28 (4 weeks) post infection with *Coxiella burnetii*. Normal sera from uninfected mice were used as negative controls.

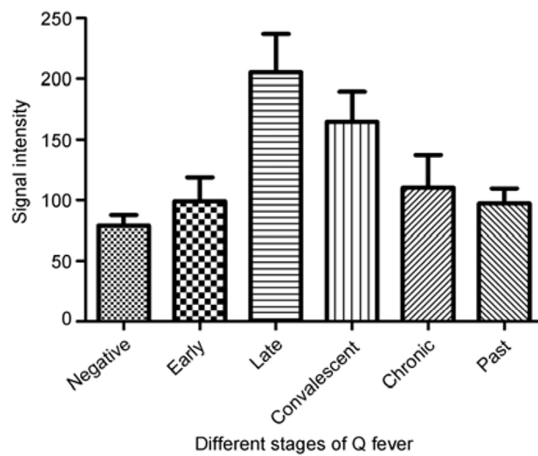

Figure 2 Overview of seroreactive proteins probed with sera from patients at different stages of Q fever infection. The *x*-axis indicates the different stages of Q fever, and the *y*-axis indicates the Ln-transformed FI values of each group of patients' sera.

tested sera are presented in Table S3. The average FI value of the proteins probed with sera from Q fever patients in their convalescent phase remained significantly higher than that probed with QAb-negative sera ($P<0.05$), but generally lower than that probed with sera taken during the late stage of acute Q fever infection (Figure 2). The average FI value of the proteins probed with chronic or past Q fever patient sera was not significantly different from that probed with the QAb-negative sera ($P>0.05$), but the FI values of certain individual proteins, particularly heat shock protein B (HspB), were markedly higher than that probed with QAb-negative sera (Table 1).

The FI mean and standard deviation (SD) of each protein probed with QAb-negative and QAb-positive sera were calculated. Proteins were considered to be positively seroreactive when the FI mean value for the QAb-positive sera was 2SD above that of the QAb-negative sera. Using these criteria, seven proteins, HspB, Ank4, Ank2, ApaH, EnhA, IcmO, and BipA, were positively recognized by more than 45% of QAb-positive sera from patients with late stage acute Q fever. The positive frequency of HspB probed with sera from patients with late stage acute Q fever was 85%. The FI value of HspB probed with sera from patients with early stage acute Q fever and chronic Q fever was over 100- and 200-fold higher, respectively, than that probed with the QAb-negative sera (Table 1).

2.4 Analysis of recombinant proteins with paired acute Q fever patient sera

The seven major seroreactive proteins of *C. burnetii* recognized by sera of patients with Q fever were analyzed with two groups of paired sera from patients with acute Q fever. In the first group examined, each patient had an acute specimen that was QAb-negative and a convalescent specimen that was QAb-positive, showing seroconversion (Figure 3A). In the second group, each patient had both an acute

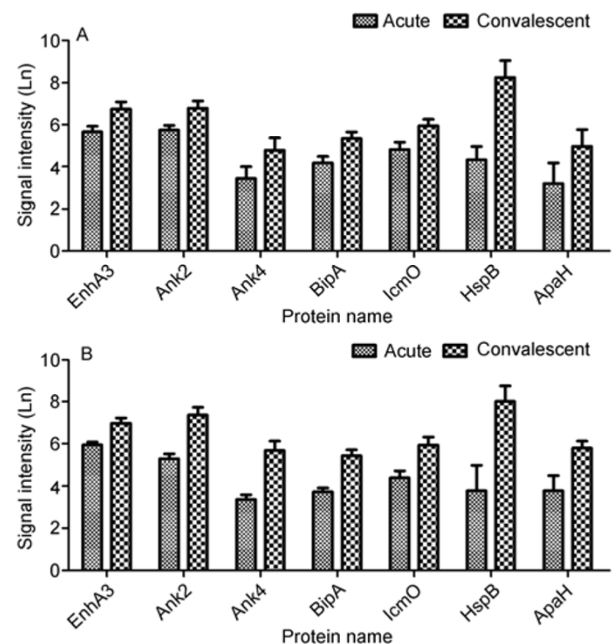

Figure 3 *Coxiella* major seroreactive proteins probed with paired Q fever patient sera. The convalescent sera were collected from the patients 5–22 days after the first sampling. A, In the first group, each protein was probed with seven pairs of acute (QAb-negative) and convalescent (QAb-positive) sera. B, In the second group, each protein was probed with seven pairs of acute (QAb-positive) and convalescent (QAb-positive) sera. The average FI of each protein probed with the convalescent sera was significantly higher than that of the acute sera ($P<0.05$).

Table 1 The average FI of each major seroreactive protein on the array probed with sera of patients with Q fever[a)]

Gene ID Protein	Sera of patients with acute Q fever							Sera of patients with			
	Ab-negative	Early stage	n=10	Late stage	n=20	Convalescent	n=7	Chronic Q fever	n=9	Past Q fever	n=14
CBU1718 HspB	12	1819	4	2367	17	1749	3	2698	6	276	5
CBU0447 Ank4	43	44	2	380	12	114	4	67	2	44	2
CBU1987 ApaH	52	84	2	265	10	157	2	99	2	23	1
CBU0884 BipA	181	132	0	473	9	295	3	104	0	154	2
CBU0201 Ank2	398	307	3	1410	9	1114	4	480	2	687	2
CBU0053 EnhA3	362	499	1	885	9	618	4	344	1	387	1
CBU1632 IcmO	97	56	0	261	9	213	4	124	1	114	3
Average	164	420	1.7	863	10.7	609	3.4	559	2	241	2.3

a) "n" indicates the number of the serum samples used in probing.

and convalescent specimen that was QAb-positive (Figure 3B). The relative FI values were analyzed using an exact Wilcoxon signed rank test. The result showed that the FI value of each of the seven proteins probed with acute sera were significantly lower than those of the convalescent sera ($P<0.05$) (Figure 3, Table S4).

3 Discussion

The availability of the complete genome sequence of *C. burnetii* has contributed significantly to our understanding of the biology and pathogenesis of this organism [13]. Assays based on two-dimensional gel resolution of endogenous antigens are able to identify seroreactive proteins of *C. burnetii* [22–24], but the lack of equal antigen representation in the organism and the denaturation of proteins during the procedure restrict the usefulness of this method for identification of these proteins. One hundred and fifty-six genes that encode proteins with potential roles in adhesion, invasion, intracellular trafficking, host modulation, detoxification, or other virulence-related functions were selected in this study [13]. The recombinant virulence-associated proteins were used to develop a subgenomic protein array of *C. burnetii*. The *Coxiella* proteins on the array were probed with sera from mice experimentally infected with *C. burnetii* and Q fever patient sera. These sera have collectively allowed novel seroreactive antigens to be identified.

The proteins on the array were screened with mouse Q fever sera from different time points after *C. burnetii* infection. An increase in reactivity of some of these proteins was observed with convalescent bleeds. This result showed that these proteins had the ability to evoke a humoral immune response and yield specific antibodies whose reactivity increased gradually with the progression of the infection. In addition, 15 proteins on the array had higher FI values than the *Coxiella* outer membrane protein 1 (Com1), which is the first surface protein antigen of *C. burnetii* recognized by Q fever sera [21]. This suggests that these proteins might be major seroreactive antigens of *C. burnetii*.

It is possible that any given response in mice may not occur in precisely the same way as in humans because of significant differences between mouse and human immunology [25]. Therefore, it was necessary to probe these proteins with human Q fever sera to understand the humoral immune responses induced by the virulence-associated proteins in Q fever patients. The *Coxiella* proteins on this array were probed with patient sera from different stages of infection, including acute (early, late and convalescent), chronic and past Q fever. The average FI value of the proteins probed with late stage acute Q fever patient sera was significantly higher than that probed with early stage acute Q fever patient sera. This indicated that the level of specific antibodies to most of the virulence-associated proteins rose markedly as the acute disease progressed to the later stage. Although the FI values of these proteins on the array generally declined with sera from the convalescent stage compared with the late stage sera, the average FI value remained significantly higher than that of the QAb-negative sera. This result indicates ongoing persistence of these antibodies during the course of acute infection.

The average FI value of the proteins probed with either chronic or past Q fever sera was not significantly different from that probed with QAb-negative sera. However, the FI values of some individual proteins, particularly HspB, were significantly higher than those of the QAb-negative sera. In addition, a further six recombinant proteins, Ank4, Ank2, ApaH, EnhA, IcmO, and BipA, were all recognized by both patient and mouse sera. These antigens may therefore be useful as potential diagnostic and vaccine candidates. To further characterize the major seroreactive antigens, the seven proteins were analyzed using paired Q fever sera. The FI value of each protein probed with the first serum was significantly lower than that probed with the second serum from the same patient, which suggested that these seven *Coxiella* proteins can stimulate the human immune responses to generate high levels of specific antibodies.

In our previous immunoproteomic analysis [26], only HspB was recognized as a major seroreactive antigen. We postulate that the six additional proteins identified in the current study are less abundant in the bacterial cells than those identified in our previous study. This may explain why they were not recognized by the human Q fever sera in immunoblot analysis. In addition, five seroreactive antigens (one hypothetical protein (CBU0891), OmpH (CBU0612), YajC (CBU1143), SucB (CBU1398), and LemA (CBU0545)) of *C. burnetii* were identified previously in two protein microarrays containing 84% and 75% of the ORFs of *C. burentii* [27,28]. These antigens were not included in this study but should be included in any future work.

HspB is located in the bacterial surface and periplasm, and is a member of the surface-associated Hsp60 family that is involved in bacterial virulence [29,30]. HspB of *C. burnetii* was previously characterized [31] and recognized as an immunodominant antigen [6,26] and as an immunodiagnostic antigen of ruminant coxiellosis [32]. In this study, the frequencies of positive reactivity to HspB probed with convalescent and chronic Q fever patient sera were markedly higher than that reported previously. Notably, the average FI values of HspB probed with sera from patients with acute and chronic Q fever were both significantly higher than QAb-negative sera. These results strongly suggest that HspB is an excellent molecular marker for serodiagnosis in both acute and chronic Q fever.

Ankyrin repeat domain proteins (Anks) are included in a protein family that shows remarkable heterogeneity amongst different isolates of *C. burnetii*. They are translocated into the host cytosol in a Dot/Icm-dependent fashion to modulate diverse functions in host cells [33]. In this study, Ank4 Ank2, Ank11, and Ank6 were recognized at

various frequencies by the late stage acute Q fever patient sera, which indicated that the patients either lacked, or had a variable response to, a variety of Ank proteins. ApaH is a diadenosine tetraphosphatase associated with bacterial fimbrial biosynthesis, carbohydrate utilization, catabolite (CRP-cAMP) regulation and cell division and probably affects virulence phenotypes such as invasion and adhesion [34,35]. BipA is a tyrosine-phosphorylated GTPase that mediates interactions between enteropathogenic *E. coli* (EPEC) and epithelial cells [36]. EnhA, a type IV secretion-independent virulence factor, has an invasion-associated trait because it contains multiple copies of the protein-protein interaction domain Sel-1 that affects the initial encounter with host cells [37]. IcmO belongs to the Icm/Dot pathogenesis systems of *C. burnetii*. These virulence-associated proteins were recognized by more than 45% of sera from patients with late stage acute Q fever.

When the proteins were analyzed in combination, 95% of patients with late stage Q fever responded to one or more of the seven major seroreactive antigens, suggesting that they are potential serodiagnostic markers of Q fever. However, their sensitivity and specificity in actual diagnosis and their specific protective efficacy in animal models still need to be evaluated further to determine their suitability as serodiagnostic and subunit vaccine antigens of Q fever.

4 Conclusion

A subgenomic protein array was developed with 101 assumed virulence-associated proteins of *C. burnetii.* The array was probed with sera from mice experimentally infected with *C. burnetii*. Sixteen proteins were recognized as major seroreactive proteins. Seven of the major immunoreactive proteins, HspB, Ank4, Ank2, ApaH, EnhA, IcmO, and BipA, were recognized by at least 45% of sera from patients with the late stage acute Q fever. The dominance of HspB as a major seroreactive protein was confirmed.

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Supporting Information

Table S1 Virulence-associated genes of *C. burnetii* amplified in this study

Table S2 Oligonucleotide primer pairs with specific restriction enzyme sites were designed for amplification of the selected virulence-associated genes of C. *burnetii*

Table S3 Average FI of each protein on the array probed with sera of patients with Q fever

Table S4 *Coxiella* major seroreactive proteins probed with paired Q fever patient sera

The supporting information is available online at life.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.