SCIENCE CHINA
Life Sciences

• RESEARCH PAPERS •

September 2011 Vol.54 No.9: 880–887
doi: 10.1007/s11427-011-4218-2

Identification of immunoreactive proteins of *Brucella melitensis* by immunoproteomics

ZHAO ZhongPeng[1†], YAN Fang[2†], JI WenHui[2], LUO DeYan[1], LIU Xin[1], XING Li[1], DUAN YueQiang[1], YANG PengHui[1], SHI XiuMin[2], LI Zhong[2] & WANG XiLiang[1*]

[1]*State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China;*
[2]*College of Animal Science and Veterinary Medicine, Shanxi Agricultural University, Taigu 030801, China*

Received March 27, 2011; accepted July 20, 2011

Infection with *Brucella* causes brucellosis, a chronic disease in humans, which induces abortion and sterility in livestock. Among the different *Brucella* species, *Brucella melitensis* is considered the most virulent and is the predominant species associated with outbreaks in China. To date, no safe human vaccine is available against *Brucella* infection. The currently used live vaccines against *Brucella* in livestock induce antibodies that interfere with the diagnosis of field infection in vaccinated animals, which is harmful to eradication programs. However, there is as yet no complete profile of immunogenic proteins of *B. melitensis*. Towards the development of a safer, equally efficacious, and field infection-distinguishable vaccine, we used immunoproteomics to identify novel candidate immunogenic proteins from *B. melitensis* M5. Eighty-eight immunoreactive protein spots from *B. melitensis* M5 were identified by Western blotting and were assigned to sixty-one proteins by mass spectrometry, including many new immunoreactive proteins such as elongation factor G, F0F1 ATP synthase subunit beta, and OMP1. These provide many candidate immunoreactive proteins for vaccine development.

***Brucella*, immunoproteomics, immunoreactive protein**

Citation: Zhao Z P, Yan F, Ji W H, *et al*. Identification of immunoreactive proteins of *Brucella melitensis* by immunoproteomics. Sci China Life Sci, 2011, 54: 880–887, doi: 10.1007/s11427-011-4218-2

Brucella are Gram-negative, nonspore-forming, nonmotile, and nonencapsulated coccobaccilli that occasionally cause brucellosis, also known as undulant fever, a chronic and debilitating febrile disease in humans that frequently induces abortion and sterility in domestic and wild animals. Human infection can result from either occupational contact or ingestion of contaminated food [1]. The incidence of human and animal brucellosis worldwide has increased rapidly since 1995. In China, *B. melitensis* is the predominant strain associated with outbreaks [2].

Based on pathogenicity and host preference, eight species have been identified within the genus: *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. cetacea*, *B. pinnipedia*, *B. neotomae*, and *B. ovis* [3]. Humans can become infected with *B. melitensis*, *B. abortus*, *B. suis*, and rarely, *B. canis*, *B. pinnipedia*, and *B. cetacea*. Among the different *Brucella* species, *B. melitensis* is considered the most virulent.

In livestock, brucellosis used to be partly controlled by conventional vaccines. Live, attenuated vaccines such as M5 and S2 have been used in animals in China; however, they are considered unsafe for humans as they can induce abortion in pregnant animals [2]. Development of the next generation of vaccines that are free from the drawbacks of the conventional vaccines requires the identification of *Brucella* antigens that can elicit a protective immune response [4].

The diagnosis of brucellosis is mainly based on the de-

†Contributed equally to this work
*Corresponding author (email: xiliangw@126.com)

© The Author(s) 2011. This article is published with open access at Springerlink.com

tection of anti-lipopolysaccharide antibodies, and the currently used live vaccines against *Brucella* in livestock induce high amounts of anti-lipopolysaccharide antibodies that interfere with the diagnosis of field infection in vaccinated animals [5]. Hence, the development of diagnostic assays based on more specific protein antigens is essential. It is very important that the live vaccines are revised to target a specific diagnostic protein antigen, which can distinguish field infection and have no effects on their protective efficacy in vaccinated animals.

Unfortunately, a definitive profile of immunogenic proteins, especially the *Brucella* protective proteins, is not yet complete [6,7]. However, much of this information can be derived from immunoproteomics, a technique that shows promise for diagnostics and vaccine development [8,9]. Moreover, the sequencing and annotation of pathogen genomes paves the way for the identification of immunogenic proteins [10–12]. Initial work describing the *Brucella* proteome and immunoproteomics has been reported. A global proteomic analysis of whole cells of *B. melitensis* 16M [13] and a comparative proteomic analysis of Rev 1 and 16M [14] have been performed; recently, immunoreactive soluble proteins of *B. melitensis* 16M have been identified by immunoproteomics using the sera of patients suffering from acute brucellosis [15]. Proteins that are located in the outer membrane of the cell envelope are of special interest since they can modify the host cell environment and mediate host cell-bacterial interactions [16]. Accumulated data have indicated that the major protective antigens appear to be components of the cell envelope [6]. Here, using bovine anti-*Brucella* sera, the whole-cell soluble proteins and membrane proteins (MPs) were screened, and 88 protein spots from *B. melitensis* M5 were identified to be immunoreactive.

1 Materials and methods

1.1 Bacterial cell culture

B. melitensis M5, a biotype I vaccine strain, was used. Stock samples stored at −70°C were inoculated in Tryptic Soy Broth (Becton Dickinson Company, Sparks, USA) and incubated to stationary growth phase by vigorous shaking for 16 h at 37°C.

1.2 Isolation of whole-cell soluble proteins [17]

Cells from 200-mL cultures in the stationary growth phase were harvested by centrifugation at 4000×*g* for 15 min at 4°C. The pellets were washed twice with low-salt washing sample buffer (3 mmol L^{-1} KCl, 1.5 mmol L^{-1} KH_2PO_4, 68 mmol L^{-1} NaCl, 9 mmol L^{-1} NaH_2PO_4) and resuspended in sonication buffer (8 mol L^{-1} urea, 1% dithiothreitol, 4% CHAPS, 100 U Benzonase, and a tablet of complete protease inhibitor cocktail) and then sonicated on ice. The solution was kept at room temperature for 1 h and centrifuged at 12000×*g* for 1 h. The supernatant was collected and stored at –70°C.

1.3 Isolation and purification of MPs [18]

After washing, harvested cells were resuspended in 100 mL buffer I (15 mmol L^{-1} Tris-HCl pH 8.0, 0.45 mol L^{-1} sucrose, 8 mmol L^{-1} EDTA, and 0.4 mg mL^{-1} lysozyme). The samples were incubated for 4 h at 4°C, centrifuged at 8000×*g* for 15 min, and resuspended in 3 mL buffer II (50 mmol L^{-1} Tris-HCl pH 7.4 and a tablet of complete protease inhibitor cocktail). The samples were chilled on ice and sonicated. Then the samples were centrifuged twice at 3000×*g* for 15 min to remove unbroken cells. Chilled 0.1 mol L^{-1} Na_2CO_3, pH 11.0 (27 mL) was added to the supernatants, and they were vortexed and immersed in ice water for 1 h. The supernatants were then subjected to ultracentrifugation at 50000×*g* for 1 h. The pellets were resuspended in 20 mL buffer II and ultracentrifuged again at 50000×*g* for 1 h. The pellets were resuspended in sample buffer (8 mol L^{-1} urea, 1% dithiothreitol, 4% CHAPS, and a tablet of complete protease inhibitor cocktail) and stored at –70°C until required.

1.4 Protein determination

The total protein concentration was determined using a Bradford kit (Applygen Technologies, Inc., Beijing, China) with bovine serum albumin as the standard. All samples, including the standard, were dissolved in the same buffer.

1.5 Western blotting

The sample proteins (200 μg) were separated by isoelectric focusing on 18-cm, pH 4–7 linear immobilized pH gradient (IPG) strips. After 12 h of rehydration at 30 V and 20°C, the following focusing parameters were applied: 50 μA per strip, with 500, 2000, or 5000 V for 30 min, linear voltage increased from 5000 to 10000 V in 2 h, then maintained at 10000 V for 8 h. After isoelectric focusing, IPG strips were equilibrated and applied onto 12% vertical slab SDS-polyacrylamide gels of 19×18.5×0.1 cm^3. Electrophoresis was performed at 20°C with a constant power supply in two steps: 20 mA/gel for 30 min then 30 mA/gel until the tracking dye reached the bottom of the gel. Gels were then stained with Coomassie Brilliant Blue R-250. The gels were scanned with an Imagescanner, and the images analyzed using version 5.0 of ImageMaster 2D Platinum software.

Proteins were transferred to polyvinylidene fluoride membranes using a TE77 semi-dry transfer unit (0.8 mA cm^{-2}, 1 h), then non-specific binding sites on the membranes were blocked for 90 min with 5% skimmed milk in Tris-buffered saline (TBS) at 37°C. Then, the membranes

were probed with a 1:1500 dilution of primary antibody for 1 h at 37°C with gentle shaking. The primary antibody was a bovine anti-*Brucella*-positive serum pool, which was a mixture of 15 bovine anti-*Brucella*-positive sera with titers of more than 1 : 1600 by tube agglutination tests. The cows had never been immunized with any *Brucella* vaccine. The control antibody was an anti-*Brucella*-negative bovine serum pool, which was a mixture of 23 anti-*Brucella*-negative bovine sera with tube agglutination titers of less than 1 : 10. The membranes were rinsed three times for 10 min each in 0.1% TBS plus Tween 20, then incubated with peroxidase-conjugated rabbit anti-bovine immunoglobulin at a dilution of 1 : 10000 in TBS containing 5% skimmed milk for 1 h at 37°C. After washing, the blots were developed using enhanced chemiluminescence Western blotting detection reagents. The specific immunoreactive protein pattern was visualized on X-ray film. Three replicate blots were used for image analysis.

1.6 *In situ* tryptic digestion, mass spectrometry (MS), and protein identification

Coomassie-stained protein spots were excised from the gel and destained with 25 mmol L^{-1} ammonium bicarbonate buffer containing 50% acetonitrile. The destained gel pieces were completely dried then rehydrated with 2 μL of 20 mmol L^{-1} ammonium bicarbonate containing 20 ng trypsin. After overnight incubation at 37°C, the gels were dried then incubated at 40°C for 1 h in 8 μL of 5% trifluoroacetic acid (TFA). The extract was transferred into a fresh microtube. A second, 1-h extraction step was performed at 30°C in 8 μL of 2.5% TFA/50% acetonitrile, then the two extracts were combined, dried, and resolubilized with 3 μL of 0.1% TFA.

Matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) MS measurements were performed on a Bruker Reflex™ III instrument (Bruker Daltonik, Bremen, Germany) operating in reflection mode. The MALDI-TOF-MS analysis was performed at 20 kV accelerating voltage and 23 kV reflecting voltage. The peptide mass fingerprints were searched against the NCBInr database using the Mascot search engine (http://www.matrixscience.com/search_form_select.html). The search parameters used were: maximum of one missed cleavage by trypsin; oxidation of methionine; carbamidomethyl modification of cysteine; charged state of +1; and mass tolerance of ±0.1 Da. Probability-based MOWSE scores greater than 75 were considered significant (P<0.05). For unambiguous identification of proteins, more than five peptides had to be matched and the sequence coverage had to be greater than 15%.

2 Results

Typical gel maps of the whole-cell soluble proteins and MPs are presented in Figures 1A and 2A. A total of 725 and 426 protein spots were detected for the whole-cell soluble proteins and MPs, respectively. Of these, 67 and 21 spots, respectively, were immunoreactive with the bovine anti-*Brucella*-positive serum pool compared with the control anti-*Brucella*-negative serum pool (data not shown). These spots were named S1 to S67 and O1 to O21, respectively (Figures 1B and 2B, respectively).

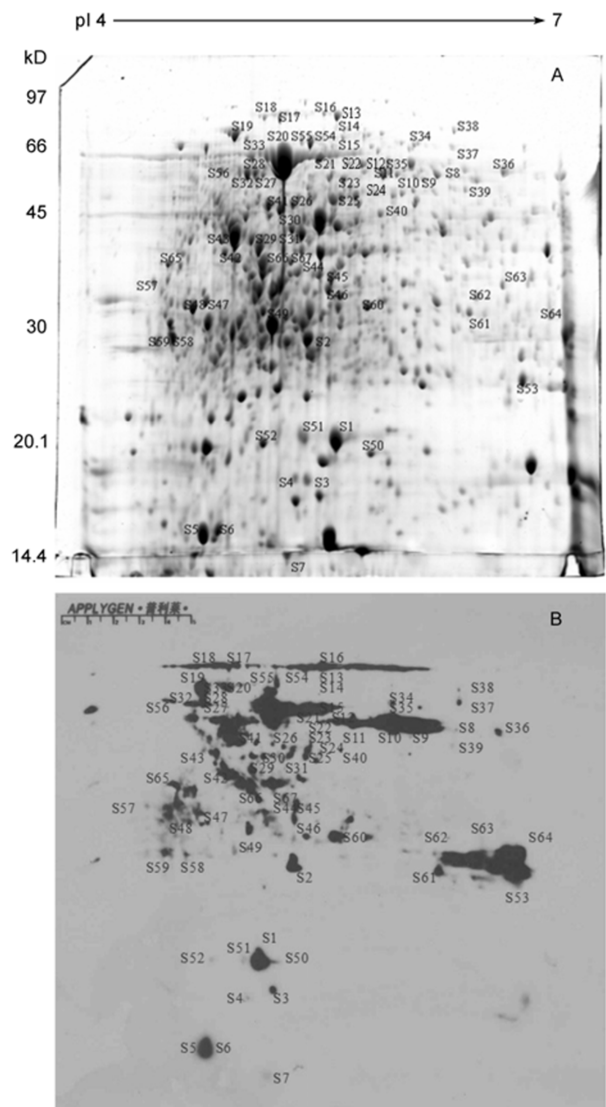

Figure 1 The proteome and Western blot of soluble proteins of *B. melitensis* M5 in the pH range 4.0–7.0. A, Soluble proteins (200 μg) were focused with IPG strips and run on 12% SDS-polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R250 and scanned. B, The primary antibody was a 1:1500 dilution of a bovine anti-*Brucella*-positive serum pool. The immunoreactive spots detected by Western blotting are marked. No spots were positive for the control primary antibody.

Seventy-nine proteins, representing the products of 61 genes, were successfully identified. Tables 1 and 2 list the identified proteins from the whole-cell soluble protein and MP samples, respectively. According to TIGR function categories, these immunoreactive proteins are mainly involved in protein fate (10/61), transport and binding (8/61),

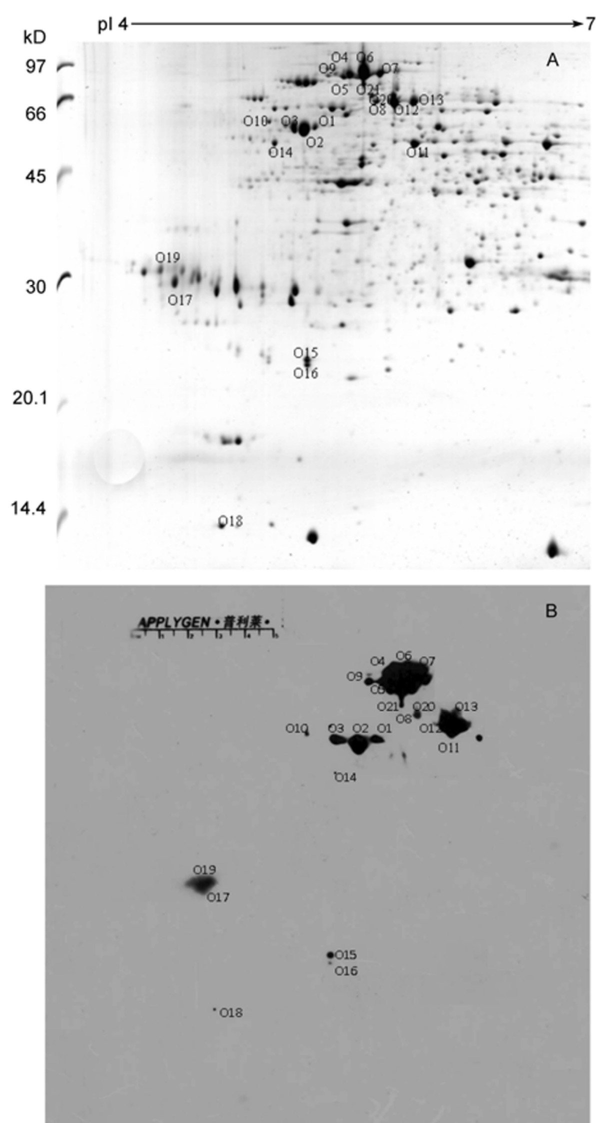

Figure 2 The proteome and Western blot of MPs of *B. melitensis* M5 in the pH range 4.0–7.0. A, MPs (200 μg) were focused with IPG strips and run on 12% SDS-polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R250 and scanned. B, The primary antibody was a 1∶1500 dilution of a bovine anti-*Brucella*-positive serum pool. The immunoreactive spots detected by Western blotting are marked. No spots were positive for the control primary antibody.

energy metabolism (8/61), protein synthesis (6/61), and cellular processes (4/61). Among the 61 identified proteins, ten were theoretical cell envelope proteins. Interestingly, the immunoreactive proteins included eight known virulence-related proteins, including OMP25, trigger factor, DnaK, glucose-6-phosphate 1-dehydrogenase, SSU ribosomal protein S1P, glutamine synthetase type I, acetolactate synthase 3 catalytic subunit, and threonine synthase [19].

3 Discussion

There is a pressing need to develop novel vaccines and specific diagnostic assays to reduce the high incidence of brucellosis worldwide. In this study, we used an immunoproteomic screen to identify many candidate *B. melitensis* antigens for these purposes.

Using immunoproteomics greatly improves the chances of discovering immunogenic proteins. We identified 61 immunoreactive proteins in this study, many more than the 32 proteins that were discovered in early protein studies using Edman sequencing or Western blotting before the genomes of the *Brucella* species had been completely sequenced [7]. To further improve the chances of identifying novel antigens, we carried out sub-proteomics. Besides the four common antigens, the MP sample contained eight antigens that were not identified in the whole-cell soluble protein sample; this indicates that sub-proteomics can be used to decrease the complexity of the whole-cell soluble protein sample to improve the discovery of novel antigens [20].

Immunoproteomics has several drawbacks such that it cannot be used to identify all the immunoreactive proteins of *Brucella*. For example, the *Brucella* lumazine synthase protein has never been identified by immunoproteomics, but is known to be able to elicit an immune and protective response against *Brucella* [21]. More importantly, different research groups have discovered very different spectra of immunoreactive proteins using immunoproteomics, because of the different protein samples and anti-*Brucella* sera used. For example, using an unabsorbed anti-*B. abortus* 1119-3 killed whole-cell rabbit serum, Al Dahouk et al. [5] identified 17 immunoreactive protein spots out of the 383 protein spots of *B. abortus* 1119-3, which were assigned to six proteins. In contrast, Teixeira-Gomes et al. [22] were able to identify 82 immunoreactive protein spots in a comparable protein sample preparation of *B. ovis* using the serum of a naturally infected ram. In another study, using antiserum collected from cows and a human accidentally infected with *Brucella*, 54 immunoreactive protein spots out of 332 protein spots in *B. abortus* CE were identified, which were assigned to 18 proteins [6]. Recently, 32 immunoreactive protein spots assigned to 27 proteins among the soluble proteins of *B. melitensis* 16M were identified using the sera of patients suffering from acute brucellosis [15]. In this study, we identified 88 immunoreactive protein spots assigned to 61 proteins out of 1151 protein spots. The higher number of proteins we identified suggests that using an anti-*Brucella*-positive serum pool can improve the chances of identification. Of the 61 immunoreactive proteins in our study, only 4, 5, 9, and 3 proteins are in common with those identified by Al Dahouk et al. [5], Teixeira-Gomes et al. [22], Connolly et al. [6], and Yang et al. [15], respectively. Therefore, all the proteins identified by immunoproteomics need further validation and evaluation by other groups and methods. Of course, live *Brucella* dynamically adjusts its protein expression profile for survival in the host and it may change enormously during the course of infection [23]. Some proteins may not be expressed under laboratory con-

Table 1 Immunoreactive soluble proteins from *B. melitensis* M5 as determined by Western blotting and MALDI-TOF-MS

Spot No.[a)]	Protein identification	Locus	MOWSE score[b)]	Sequence coverage (%)	Peptides matched
Cell envelope					
S2	OMP28/BP26	BMEI0536	75	40	6
S10	UDP-N-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase	BMEI0574	130	28	9
S12, S15	OMP1	BMEI0830	125, 137	32, 26	17, 14
S60	31 kD immunogenic protein precursor	BMEI0796	86	22	6
S65	Porin	BMEI1305	83	31	7
S58	OMP25c	BMEI1829	75	35	6
Cellular processes					
S1, S51	DNA protection during starvation protein	BMEI1980	109, 92	36, 44	6, 7
S9	Branched-chain alpha-keto acid dehydrogenase subunit E2	BMEII0746	116	25	9
S14	Cell division protein FtsZ	BMEI0585	204	24	9
S64	Cell division inhibitor minD	BMEII0926	89	35	8
Protein fate					
S18	Molecular chaperone DnaK	BMEI2002	125	15	9
S57	DnaK	BMEI1549	223	25	16
S19, S45	Chaperonin GroEL	BMEII1048	210, 174	15, 28	9, 18
S20	HtrA	BMEI1330	137	19	8
S26	Acriflavin resistance protein A precursor	BMEI1630	174	21	7
S35	Leucyl aminopeptidase	BMEI1261	139	23	9
S48	GrpE protein	BMEI1777	126	31	7
S50	Peptidyl-prolyl cis-trans isomerase A	BMEI0888	138	39	6
S59	Protein-L-Isoaspartate O-methyltransferase	BMEI1030	180	24	6
Transport and binding proteins					
S28	Periplasmic oligopeptide-binding protein precursor	BMEII0735	214	27	14
S29	Metal chelate periplasmic binding protein	BMEI0658	155	29	8
S30	Trehalose/maltose-Binding protein	BMEI1716	89	21	6
S31	Leu/Ile/Val-binding protein precursor	BMEII0103	137	28	7
S32	Periplasmic oligopeptide-binding protein precursor	BMEII0735	88	25	10
S42	Putrescine-binding protein, putative	BMEI0411	103	24	6
S43	Iron(III)-binding periplasmic protein precursor	BMEII1120	128	59	19
S67	D-galactose-binding periplasmic protein precursor	BMEII0983	135	49	13
Energy metabolism					
S3	Lactoylglutathione lyase	BMEI0730	108	41	7
S8	Glucose-6-phosphate 1-dehydrogenase	BMEII0513	217	30	13
S11	F0F1 ATP synthase subunit beta	BMEI0251	239	36	12
S36	F0F1 ATP synthase subunit alpha	BMEI0249	148	23	11
S38	Transketolase	BMEI 0311	223	28	22
S41	Enolase	BMEI0851	147	20	8
S47	Electron transfer flavoprotein, alpha subunit	BMEI0097	75	49	5
S13	Acetyl/propionyl-CoA carboxylase alpha chain	BMEI1925	281	58	19
Central intermediary metabolism					
S46	Putative thiosulfate sulfurtransferase	BMEI0931	175	19	9
S61	2,5-diketo-D-gluconic acid reductase	BMEII1060	168	52	15
DNA metabolism					
S52	DnaK suppressor protein homolog	BMEI0949	85	45	5
Protein synthesis					
S16	Elongation factor G	BMEI0754	174	24	13
S27	Histidyl-tRNA synthetase	BMEII1056	135	27	11

(*To be continued on the next page*)

(*Continued*)

Spot No.[a]	Protein identification	Locus	MOWSE score[b]	Sequence coverage (%)	Peptides matched
S53	Ribosome recycling factor	BMEI0826	95	41	6
S55	SSU ribosomal protein S1P	BMEI1915	116	16	9
S66	Protein translation elongation factor Ts (EF-Ts)	BMEI0824	157	64	16
Amino acid biosynthesis					
S21	Glutamine synthetase type I	BMEI0979	213	25	11
S34	Acetolactate synthase 3 catalytic subunit	BMEI0617	189	21	9
S24	Threonine synthase	BMEI1450	179	57	15
Biosynthesis of cofactors, prosthetic groups and carriers					
S40	GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase	BMEI1505	145	42	17
S62	2,3,4,5-tetrahydropyridine-2-carboxylateN-succinyltransferase	BMEII0270	99	16	5
Fatty acid and phospholipid metabolism					
S23	3-oxoacyl-(acyl carrier protein) synthase II	BMEI1473	136	51	13
S39	Acetyl-CoA carboxylase biotin carbox-ylase	BMEI1063	157	39	12
S63	Acetyl-CoA carboxylase carboxyltransfer-ase subunit alpha	BMEI0039	152	34	9
Transcription					
S17	Polynucleotide phosphory-lase/polyadenylase	BMEI1961	183	17	9
Unclassified					
S37	Electron transfer flavoprotein-ubiquinone oxidoreductase precursor	BMEI1320	218	31	16
Not determined					
S4, S5, S6, S7, S22, S25, S49, S54, S56					

a) Spot numbers refer to the numbers on the gel shown in Figure 1A. b) Protein scores greater than 75 are significant (P<0.05).

Table 2 Immunoreactive MPs from *B. melitensis* M5 as determined by Western blotting and MALDI-TOF-MS

Spot No.[a]	Protein identification	Locus	MOWSE score[b]	Sequence coverage (%)	Peptides matched
Cell envelope					
O5, O6, O7, O21	OMP1	BMEI0830	114, 177 193, 197	46, 38 54, 51	36, 20 43, 42
O8, O12	Iron-regulated outer membrane protein FRPB	BMEII0105	153, 180	42, 45	30, 31
O13	Bacterial surface antigen (D15)	BMEI1895	218	77	40
O15, O16	OMP25	BMEI1249	83, 101	38, 62	12, 16
O17, O19	OMP25b	BMEI1007	76, 93	25, 25	5, 5
Protein fate					
O1, O2, O3	Chaperonin GroEL	BMEII1048	175, 128 108	40, 43 35	22, 23 17
O10	Trigger factor	BMEI1069	82	41	23
Energy metabolism					
O11	ATP synthase subunit B	BMEI0251	128	67	28
O20	Acetyl/propionyl-CoA carboxylase Alpha chain	BMEI1925	93	38	26
Protein synthesis					
O18	50S ribosomal protein	BMEI0748	61	58	5
Cell envelope biogenesis					
O4, O9	Organic solvent Tolerance protein	BMEI1264	168, 150	51, 51	39, 42
Unknown					
O14	Hypothetical protein	BMEI0178	85	65	11

a) Spot numbers refer to the numbers on the gel shown in Figure 2A. b) Protein scores greater than 75 are significant (P<0.05).

ditions. Furthermore, even under common laboratory conditions, the protein expression profile of *B. melitensis* is different from that of *B. abortus* [24]. The production of antibodies directed against specific proteins may be host-specific. Some proteins are highly immunogenic in one type of animal but fail to show any immunogenicity in other animal

types. Anti-OMP28 (BP26) antibodies were detected in *Brucella*-infected humans and goats, but not in pigs and cattle [25]. Additionally, the kinetics of the serum antibody response to *Brucella* proteins may reflect the different stages of infection. Antibodies to BP26 were found only in the sera of patients suffering from acute brucellosis [26].

The complete profile of immunoreactive proteins will ultimately help to understand the interesting biology of different *Brucella* species, such as their virulence and host preference. Considering our data and those of Connolly et al. [6], it is likely that the presence of ribosomal proteins in the membrane preparations from prokaryotes genuinely indicates their localization at the membrane, rather than contamination by cytosolic proteins [27,28]. OMP25 belongs to the OMP A protein family, which accounts for 30%–40% of the outer membrane. In the genome of *B. melitensis*, four *OMP25* genes are predicted: BMEI1249, BMEI1007, BMEI1829, and BMEI1830, of which three (BMEI1249, BMEI1007, and BMEI1829) were identified in this study. Importantly, OMP25 is an immune response regulator and may mediate inhibition of the host cell production of tumor necrosis factor alpha [29]. Mutants of *B. melitensis*, *B. abortus*, and *B. ovis* strains with the *OMP25* gene inactivated have been found to be attenuated in mice, goats, and cattle [30–32]. Heat shock proteins are vital for *Brucella* survival under various harsh conditions; these were identified in almost all immunoproteomic studies. The blockage of heat shock protein function by antibodies may be an important host protection strategy. HtrA is generally thought to serve as a stress response protease in the periplasmic space and has been shown to be important for adaption to the intracellular environment of host macrophages [33,34]. DnaK is important for *Brucella* growth and survival under stress conditions: A constitutive DnaK mutant failed to multiply in murine macrophages and was rapidly eliminated [35]. Trigger factor is an ATP-independent chaperone that binds to the nascent polypeptide chain [36]. Recently trigger factor was revealed to be a protective antigen against brucellosis [37]. In addition, the proteins involved in energy metabolism, iron acquisition [14], and protein synthesis may be vital for *Brucella* survival, some of which were also identified to be immunoreactive in this study. Recently, recombinant S-adenosyl-L-homocysteine hydrolase has been shown to stimulate the production of interferon gamma and induce a high level of protection against *B. melitensis* [15].

In summary, we discovered 61 immunoreactive proteins from among the whole-cell soluble proteins and MPs of *B. melitensis* M5. These data will help to develop novel vaccines and specific diagnostic assays in the future. At present, validation of these proteins' immunoreactivity and investigation of their protective efficacy is in progress. Twelve of them have been validated as immunogenic, including the iron-regulated outer membrane protein FRPB (BMEII0105) and grpE protein (BMEI1777). Using anti-*Brucella* sera collected from animals and humans accidentally infected with different species of *Brucella*, all the novel immunoreactive proteins of the various *Brucella* species will gradually be discovered and investigated.

We gratefully thank Jiang SenLin (CDC, Baicheng, China) for providing the serum samples and Janice Willson for assistance in preparing the manuscript. This work was supported by the National Basic Research Program of China (Grant No. 2010CB530200).

1 Corbel M J. Brucellosis: an overview. Emerg Infect Dis, 1997, 3: 213–221
2 Deqiu S, Donglou X, Jiming Y. Epidemiology and control of brucellosis in China. Vet microbiol, 2002, 90: 165–182
3 Jahans K L, Foster G, Broughton E S. The characterisation of *Brucella* strains isolated from marine mammals. Vet Microbiol, 1997, 57: 373–382
4 Khan A S, Mujer C V, Alefantis T G, *et al*. Proteomics and bioinformatics strategies to design countermeasures against infectious threat agents. J Chem Inf Model, 2006, 46: 111–115
5 Al Dahouk S, Nockler K, Scholz H C, *et al*. Immunoproteomic characterization of *Brucella abortus* 1119-3 preparations used for the serodiagnosis of *Brucella* infections. J Immunol Methods, 2006, 309: 34–47
6 Connolly J P, Comerci D, Alefantis T G, *et al*. Proteomic analysis of *Brucella abortus* cell envelope and identification of immunogenic candidate proteins for vaccine development. Proteomics, 2006, 6: 3767–3780
7 DelVecchio V G, Wagner M A, Eschenbrenner M, *et al*. *Brucella* proteomes—a review. Vet microbiol, 2002, 90: 593–603
8 Pizza M, Scarlato V, Masignani V, *et al*. Identification of vaccine candidates against serogroup *B. meningococcus* by whole-genome sequencing. Science, 2000, 287: 1816–1820
9 Rappuoli R. Reverse vaccinology, a genome-based approach to vaccine development. Vaccine, 2001, 19: 2688–2691
10 Chain P S, Comerci D J, Tolmasky M E, *et al*. Whole-genome analyses of speciation events in pathogenic Brucellae. Infect immun, 2005, 73: 8353–8361
11 Paulsen I T, Seshadri R, Nelson K E, *et al*. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc Natl Acad Sci USA, 2002, 99: 13148–13153
12 DelVecchio V G, Kapatral V, Redkar R J, *et al*. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. Proc Natl Acad Sci USA, 2002, 99: 443–448
13 Wagner M A, Eschenbrenner M, Horn T A, *et al*. Global analysis of the *Brucella melitensis* proteome: Identification of proteins expressed in laboratory-grown culture. Proteomics, 2002, 2: 1047–1060
14 Eschenbrenner M, Wagner M A, Horn T A, *et al*. Comparative proteome analysis of *Brucella melitensis* vaccine strain Rev 1 and a virulent strain, 16M. J Bacteriol, 2002, 184: 4962–4970
15 Yang Y, Yin J, Guo D, *et al*. Immunization of mice with recombinant S-adenosyl-L-homocysteine hydrolase protein confers protection against *Brucella melitensis* infection. FEMS Immunol Med Microbiol, 2011, 61: 159–167
16 Celli J, Gorvel J P. Organelle robbery: *Brucella* interactions with the endoplasmic reticulum. Curr Opin Microbiol, 2004, 7: 93–97
17 Peng X, Ye X, Wang S. Identification of novel immunogenic proteins of *Shigella flexneri* 2a by proteomic methodologies. Vaccine, 2004, 22: 2750–2756
18 Ying T Y, Wang J J, Wang H L, *et al*. Immunoproteomics of membrane proteins of *Shigella flexneri* 2a 2457T. World J Gastroenterol, 2005, 11: 6880–6883
19 Delrue R M, Lestrate P, Tibor A, *et al*. *Brucella* pathogenesis, genes identified from random large-scale screens. FEMS Microbiol Lett, 2004, 231: 1–12
20 Patton W F. Proteome analysis. II. Protein subcellular redistribution: linking physiology to genomics via the proteome and separation technologies involved. J Chromatogr, 1999, 722: 203–223

21 Velikovsky C A, Goldbaum F A, Cassataro J, *et al*. *Brucella* lumazine synthase elicits a mixed Th1-Th2 immune response and reduces infection in mice challenged with *Brucella abortus* 544 independently of the adjuvant formulation used. Infect Immun, 2003, 71: 5750–5755
22 Teixeira-Gomes A P, Cloeckaert A, Bezard G, *et al*. Identification and characterization of *Brucella ovis* immunogenic proteins using two-dimensional electrophoresis and immunoblotting. Electrophoresis, 1997, 18: 1491–1497
23 Rafie-Kolpin M, Essenberg R C, Wyckoff J H 3rd. Identification and comparison of macrophage-induced proteins and proteins induced under various stress conditions in *Brucella abortus*. Infect Immun, 1996, 64: 5274–5283
24 Eschenbrenner M, Horn T A, Wagner M A, *et al*. Comparative proteome analysis of laboratory grown *Brucella abortus* 2308 and *Brucella melitensis* 16M. J Proteome Res, 2006, 5: 1731–1740
25 Lindler L E, Hadfield T L, Tall B D, *et al*. Cloning of a *Brucella melitensis* group 3 antigen gene encoding Omp28, a protein recognized by the humoral immune response during human brucellosis. Infect Immun, 1996, 64: 2490–2499
26 Rossetti O L, Arese A I, Boschiroli M L, *et al*. Cloning of *Brucella abortus* gene and characterization of expressed 26-kilodalton periplasmic protein: potential use for diagnosis. J Clin Microbiol, 1996, 34: 165–169
27 Sinha S, Kosalai K, Arora S, *et al*. Immunogenic membrane-associated proteins of Mycobacterium tuberculosis revealed by proteomics. Microbiology, 2005, 151: 2411–2419
28 Wehmhoner D, Dieterich G, Fischer E, *et al*. “LaneSpector”, a tool for membrane proteome profiling based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis/liquid chromatography-tandem mass spectrometry analysis: application to Listeria monocytogenes membrane proteins. Electrophoresis, 2005, 26: 2450–2460
29 Jubier-Maurin V, Boigegrain R A, Cloeckaert A, *et al*. Major outer membrane protein Omp25 of *Brucella suis* is involved in inhibition of tumor necrosis factor alpha production during infection of human macrophages. Infect Immun, 2001, 69: 4823–4830
30 Edmonds M D, Cloeckaert A, Booth N J, *et al*. Attenuation of a *Brucella abortus* mutant lacking a major 25 kDa outer membrane protein in cattle. Am J Vet Res, 2001, 62: 1461–1466
31 Edmonds M D, Cloeckaert A, Elzer P H. *Brucella* species lacking the major outer membrane protein Omp25 are attenuated in mice and protect against *Brucella melitensis* and *Brucella ovis*. Vet Microbiol, 2002, 88: 205–221
32 Edmonds M D, Cloeckaert A, Hagius S D, *et al*. Pathogenicity and protective activity in pregnant goats of a *Brucella melitensis* Delta-omp25 deletion mutant. Res Vet Sci, 2002, 72: 235–239
33 Elzer P H, Phillips R W, Robertson G T, *et al*. The HtrA stress response protease contributes to resistance of *Brucella abortus* to killing by murine phagocytes. Infect Immun, 1996, 64: 4838–4841
34 Phillips R W, Elzer P H, Robertson G T, *et al*. A *Brucella melitensis* high-temperature-requirement A (htrA) deletion mutant is attenuated in goats and protects against abortion. Res Vet Sci, 1997, 63: 165–167
35 Kohler S, Ekaza E, Paquet J Y, *et al*. Induction of dnaK through its native heat shock promoter is necessary for intramacrophagic replication of *Brucella suis*. Infect Immun, 2002, 70: 1631–1634
36 Valent Q A, Kendall D A, High S, *et al*. Early events in preprotein recognition in *E. coli*: interaction of SRP and trigger factor with nascent polypeptides. EMBO J, 1995, 14: 5494–5505
37 Yang X, Walters N, Robison A, *et al*. Nasal immunization with recombinant *Brucella melitensis* bp26 and trigger factor with cholera toxin reduces *B. melitensis* colonization. Vaccine, 2007, 25: 2261–2268

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.