

## M5 and Identification of New Immunogenic Proteins

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### Abstract

*Brucellae* are facultative intracellular gram-negative bacteria that cause human disease and significant worldwide economic loss due to infection of livestock. Available vaccines against *Brucella* spp. are live attenuated *Brucella* strains. In order to engineer a better vaccine to be used in animals and humans, our laboratory aims to develop an innocuous subunit vaccine. Particularly, we are interested in *Brucella* membrane proteins (MPs). In this study, an immunoproteomic approach was utilized to identify novel antigenic candidate proteins from Chinese *Brucella* vaccine strain M5 membrane proteins. The membrane proteins were separated by 2-DE and analyzed by western-blotting for their reactivity with the antiserum obtained from goats naturally infected by *Brucella*. Of a total of 9 immunogenic proteins identified, 7 were shown to be the novel antigens for *Brucella* spp. Some of the major protein components include outer-membrane protein OMP25, OMP31, and several new immunogenic proteins were identified, such as isovaleryl coenzyme A dehydrogenase, nitroglycerin reductase, succinyl-CoA synthetase subunit and S-adenosine-L-homocysteine hydrolase. Comparing these gene sequences revealed that 8 out of 9 immunoreactive protein genes were found in all 5 different *Brucella* strains. The elucidation of the immunome of *Brucella* vaccine strain M5 MPs identified a number of candidate proteins for developing vaccines against *Brucella* infection in livestock.

**Keywords:** *Brucella*; Immunoproteomics; Vaccine candidate; 2-DE

### Introduction

Brucellosis, one of the most important re-emerging zoonoses caused by bacteria of the genus *Brucella*, can affect many mammal species and is transmissible to humans, thus having an important socio-economic impact. It now occurs in many countries and threatens almost 1/10-1/5 of the world's population [1,2]. *Brucella melitensis* is considered to be the most widespread and virulent pathogen, which frequently causes chronic and lifelong infection in many natural hosts. Once infected, abortion or infertility may result in animals, while human infection causes a disease known as undulant fever or Malta fever with severe complications including meningitis, endocarditis, spondylitis and arthritis [3].

Nowadays, *Brucella* infections in livestock are usually prevented by administration of attenuated live vaccines. These include *Brucella abortus* S19 strain, *Brucella melitensis* M5 strain and *Brucella suis* S2 strain which have been used for cattle, sheep and goats, and swine immunization in China, respectively [4,5]. The main drawback with these vaccines is that they induce O-polysaccharide-specific antibodies that interfere with the serologic diagnosis of the disease. Also, due to residual pathogenicity, none of these vaccine strains can be used in humans [6]. Because of its potential use as a biological warfare agent [7], much effort is being placed on the development of next generation vaccines strategies under development include subunit vaccines [8], overexpression of a protective antigen in a less virulent *Brucella* vaccine strain [9], vaccination with *E. coli* expressing a *Brucella* antigen [10], and DNA vaccines [11]. The identification of proteins that are able to elicit an immune response is essential for efficient vaccine development. Immunogenic *Brucella* membrane proteins are ideal vaccine candidates since they play important roles in bacterial toxicity, immune protection as well as intra and extra cellular signal transduction [12].

Proteomics has been widely applied to develop two-dimensional polyacrylamide gel electrophoresis maps and databases, evaluate gene expression profiles under different environmental conditions, assess global changes associated with specific mutations, and define drug

targets of bacterial pathogens [13]. When coupled to immunological assays, proteomics may also be used to identify B-cell and T-cell antigens within complex protein mixtures. As shown in the present study, the combination of proteomics and Western-Blotting (WB), i.e. immune proteomics, helped us identify dominant immunogens from membrane proteins that hold promise as vaccine candidates. This rapid and efficient method for identifying vaccine candidates differs from other reports in that vaccine candidates were determined by practical evaluation of immune reactivity rather than by theoretical analysis of genes and proteins based on bioinformatics [14,15]. In recent years, this methodology has been shown as effective in aiding rapid development of specific, safe, and highly efficacious vaccines for humans and livestock and about 21 types of pathogenic microorganisms were subjected to screen for antigenic proteins by using immune proteomics, which include *Helicobacter pylori* [16-18], *Aeromonas hydrophila* [10,19], *Borrelia garinii* [20], *Shigella flexneri* [2,21,22], trachoma-and pneumonia-causing *Chlamydia* [23] and *Bacillus anthracis* [24,25].

The completion of the sequencing and annotation of the genomes from *B. melitensis* [26], *B. suis* [27], and *B. abortus* [28], provides a favorable tool for a proteomic based analysis of *Brucella*. Initial work describing the *B. abortus* proteome [29,30], a global proteomic analysis of whole cells of *B. melitensis* 16M grown under laboratory conditions [31,32], and a comparative proteomic analysis between *B. melitensis* vaccine strain Rev 1 and 16M [33]. In order to identify *Brucella* antigens which can elicit protective immune response for developing

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*Brucella* subunit vaccine, Western blotting of 2-DE gels has been used with antiserum from *Brucella* -infected animals or patients [34,35].

*B. melitensis* vaccine strain M5 was developed in 1962 by the Haerbin Institute of Veterinary Medicine at Chinese Academy of Agriculture Sciences. The vaccine is derived from the virulent strain of *B. melitensis* and used mainly to vaccinate both pregnant and non pregnant sheep. Inhalation of the sprayed vaccine at a dose of five billion bacteria per animal was found to be the most effective delivery means; 1 year of protection against brucellosis was proposed. It was first used in 1964 on a trial basis and was used on a large scale beginning in 1970 and is still used in sheep in China [36].

Therefore, in this study, using antiserum collected from goats naturally infected by *B. melitensis*, 9 immunogenic proteins in the *B. melitensis* M5 vaccine strain membrane proteins were identified using 2-DE Western blot analysis. The immunogenic proteins identified here can be of great value for developing future brucellosis subunit vaccines. This case also confers a more directed approach to vaccine development. The genes of 9 candidate antigenic proteins were amplified and sequenced, the sequence differences were compared among 5 different *Brucella* species.

## Materials and Methods

### Bacterial culture

*B. melitensis* M5 vaccine strain was cultured in TSB (Tryptone Soya Broth) liquid medium at 37°C, 200 rpm for 48-72 h. When the OD<sub>600</sub> was around 2.8, the cells were collected by centrifugation at 6000 × g for 10 min at 4°C.

### Extraction of bacterial membrane proteins

Membrane protein extraction and solubilization were performed as Molloy [37] and Henningsen et al. [38] reported with modification. Briefly, bacteria were collected at 4°C, 8,000 rpm for 10 min, washed with ice PBS (3 mM KCl/1.5 mM KH<sub>2</sub>PO<sub>4</sub>/68 mM NaCl/9 mM NaH<sub>2</sub>PO<sub>4</sub>) for 4 times, resuspended in 50 mM Tris-Cl (pH 7.5) to reach a final volume of 5 ml. The cells were sonicated for 10 min on ice using a Sonifier 750 (Branson Ultrasonics Corp, Danbury, CT) with the following parameters: 2 s of sonication with a 2 s interval, 25% highest power, and centrifuged at 4°C, 2,000 g for 10 min. The supernatant was collected and mixed with 10 times' supernatant volume of ice cold 0.1 M Na<sub>2</sub>CO<sub>3</sub> (pH 11.0). The mixture was incubated in an ice bath with constant gentle disturbance for 1 h and centrifuged at 4°C, 100,000 g for 1 h. The supernatant was removed and the pellet was resuspended with 50 mM Tris-Cl (pH 7.5), centrifuged at 4°C, 100,000 g for 1 h. The supernatant was discarded, and the pellet was lysed in the lysis buffer (5 M Urea/2 M Thiourea/2% CHAPS/1% SB 3-10/1% ASB-14/1% DTT) at room temperature for 1 h and centrifuged at 100,000 g for 30 min. The supernatant was collected; the protein concentration was measured using the PlusOne 2-D Quant Kit (GE-Healthcare). 800 µg supernatant was aliquoted into each tube, which was directly subjected to one-dimensional SDS-PAGE analysis or kept at -70°C.

### Two-dimensional electrophoresis (2-DE)

**IEF:** To detect the membrane proteins with 2-DE, we followed the approach described by Görg and Dunn with a little modification [39]. Briefly, Eighteen-centimeter immobilized pH gradient (IPG) strips (pH ranges, 4 to 7) were rehydrated at room temperature in a passive rehydration tray for 20 h with 350 µl of sample containing 800 µg of total protein. Isoelectric focusing (IEF) was conducted at 20°C for 16 h. The parameters used for isoelectric focusing were: 50V 2h; 500V 1h;

1000V 1h; 3000V 3h followed by a linear increase to 8000 V for 3 h. The final phase of 8000V was terminated after 48000 Vh.

**SDS-PAGE:** After isoelectric focusing, the strips were incubated in 5 ml equilibration buffer I (50 mM Tris-Cl (pH 8.8) / 6 M Urea/30% glycerol/2% SDS/trace bromphenol/50 mg DTT) on a horizontal shaker for 15 min, followed by incubation in 5 ml equilibration buffer II (50 mM Tris-Cl (pH 8.8)/6 M Urea/30% glycerol/2% SDS/trace bromphenol/225 mg iodoacetamide) for 15 min. IPG strips and SDS-PAGE molecular weight standards (7–175 kDa; NEW ENGLAND BioLabs) were loaded into homogeneous 12.5% polyacrylamide gels and sealed with 0.5% agarose solution. The SDS-PAGE was performed in two steps at 12°C in an electrophoresis unit (Ettan™ DALT six, GE-Healthcare): 1 w/gel for around 30 min, and then at 8 w/gel until the bromphenol reached the bottom of the gels. The two twin's gels were performed at the same time. One of the twin gels was stained with colloidal Coomassie Brilliant Blue G-250, the other was subjected to western blotting. Three replicates were run. Coomassie stained gels were digitized by using an image scanner (U9909-H7L0, Amersham Biosciences).

**Immunoblotting:** Proteins from non-stain twin gels were transferred onto 0.45 µm PVDF membranes by using a Semi-Dry blot electrophoresis transfer system (Trans-Blot, Bio-Rad) with a running buffer of 25 mM Tris-HCl, 192 mM glycine and 20% methanol at 20 V for 1 h. After the transfer, the membrane was blocked in 100 ml blocking buffer (Tiangen Bio, Beijing) at 37°C for 1 h or 4°C overnight. Then the membrane was washed with TBST (0.05 mol/L Tris-HCl/1% Tween-20 pH 7.4, 0.2 mol/L NaCl) for 5 min × three times. For antibody incubation, the membrane was first incubated with the primary antibody (anti-serum from goats infected *Brucella melitensis* naturally, which was positive for Rose Bengal plate agglutination test, RBPT), at a dilution of 1:100 in TBST containing 0.1% BSA (Bovine Serum Albumin) at 37°C for 1 h and washed with TBST for 5 min × three times. The membrane was incubated with the secondary antibody (HRP conjugated rabbit anti goat IgG, Sigma), at a dilution of 1:10000 in TBST containing 0.1% BSA at 37°C for 1 h, washed with TBST for 5 min × four times, and developed with TMB (Tetramethyl Benzidine) for 5-10 min until optimum color was developed. Antiserums from uninfected goats were used as control.

**Image analysis and protein identification:** Dot pattern analysis was accomplished by using the 2-D image analysis software Delta2D (Decodon GmbH). The immunoreactivity of each spot that showed positive signal in Western blot analysis was verified through their excision from 2-D gels, followed by dot blotting. These protein dots were sent to Beijing Ruizhi Xinghua Data Co., Ltd. for tryptic in-gel digestion and LC-MS/MS. Gel plugs were placed in 96-well plates and then washed with water. Tryptic digestions were performed on a MassPrep liquid handling robot (Micromass) according to the manufacturer's specifications and using sequencing grade modified trypsin (Promega). After extraction from the gel into 50% acetonitrile/0.1% formic acid, peptides were lyophilized in a speed vacuum and resuspended in 10 µl of 0.1% formic acid solution. The "peak list" was created automatically with EXTRACTMS program provided by the Xcalibur software package (Bioworks v 3.3). The MS spectra with a total ion currency (TIC) higher than 10<sup>4</sup> were used to search for matches against NCBI (*Brucella melitensis*) protein sequence database (5924 entries) using TurboSEQUEST algorithm. The search parameters included based on the TurboSEQUEST algorithm were: i) precursor ion mass tolerance less than 2.0 amu, ii) fragment ion mass tolerance less than 1.0 amu, iii) up to 2 missed cleavages allowed, and iv) fixed

modifications by cysteine carboxyamidomethylation (plus 57.02 amu) and variable modifications by methionine oxidation (plus 15.99 amu). In accordance with the criteria describe by Link et al. [40], matched peptide sequences of identified proteins had to pass the following: i) the cross-correlation scores (Xcorr) of matches must be greater than 1.5, 2.0, and 2.5 for peptide ions of charge state +1, +2 and +3, respectively; ii)  $\Delta C_n$  values of the best peptide matches must be at least 0.1; and iii) the primary scores (Sp) must be at least 600. Protein identification required at least two distinct peptides matching these criteria. Results obtained from SEQUEST were validated using PeptideProphet [41] and ProteinProphet [42]. A ProteinProphet probability score of  $\geq 0.9$  was used, and a PeptideProphet score of  $\geq 0.95$  for every peptide in this protein was used. The degree of completeness of b- and y-ion series for each TurboSEQUEST result was manually checked for every protein identified.

**Bioinformatic retrieval:** The PSORTb version 2.0.4 was used for prediction of subcellular localization of the identified immunogenic proteins (<http://www.psort.org/>). Signal peptides were predicted using SignalIP 3.0 (<http://www.cbs.dtu.dk/services/SignalP3.0>), which was trained on data from Gram negative prokaryotes. Other information about identified proteins was searched for in KEGG data base. (<http://www.kegg.com/>) Sequences of identified proteins were submitted to a BLAST server to find similar sequences. (<http://www.ncbi.nlm.nih.gov/BLAST/>).

### PCR detection

Primers were synthesized against the corresponding translated open reading frames (ORFs) of the genes of identified proteins. The genomic DNA from *B. melitensis* 16M strain, *B. melitensis* M5 strain *B. abortus* 544A strain and *B. abortus* S19 strain were isolated with the genomic DNA extraction kit (AXYGEN™, HangZhou China). All the PCR products were inserted into PMD18-T vector (TaKaRa Bio) and sequenced (Beijing Sunbio). Alignments of the sequencing results were checked with the MegAlign in DNASTAR.

### Antigenicity analysis

The secondary structure of immunoreactive proteins mentioned above was predicted by the methods of Garnier-Robson [38], Chou-Fasman [21] and Karplus-Schulz [20] based on their amino acid sequences. And Hydrophilicity plot, Surface probability plot and Antigenic index for these proteins were obtained by the methods of Kyte [43], Emini [43] and Ameson-Wolf [17], respectively.

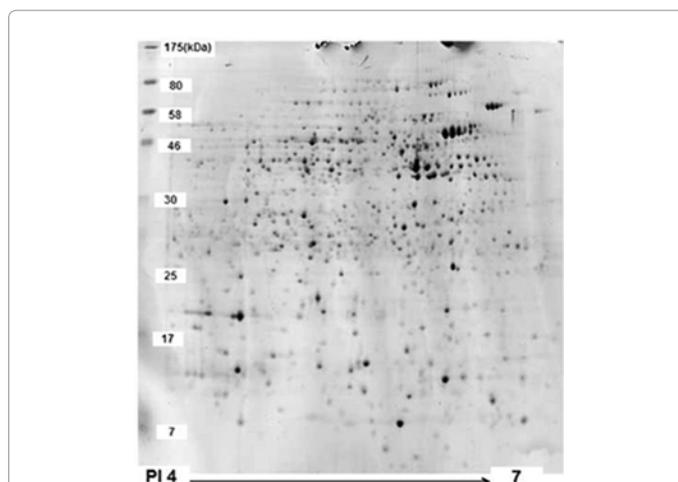
## Results

### Membrane proteome

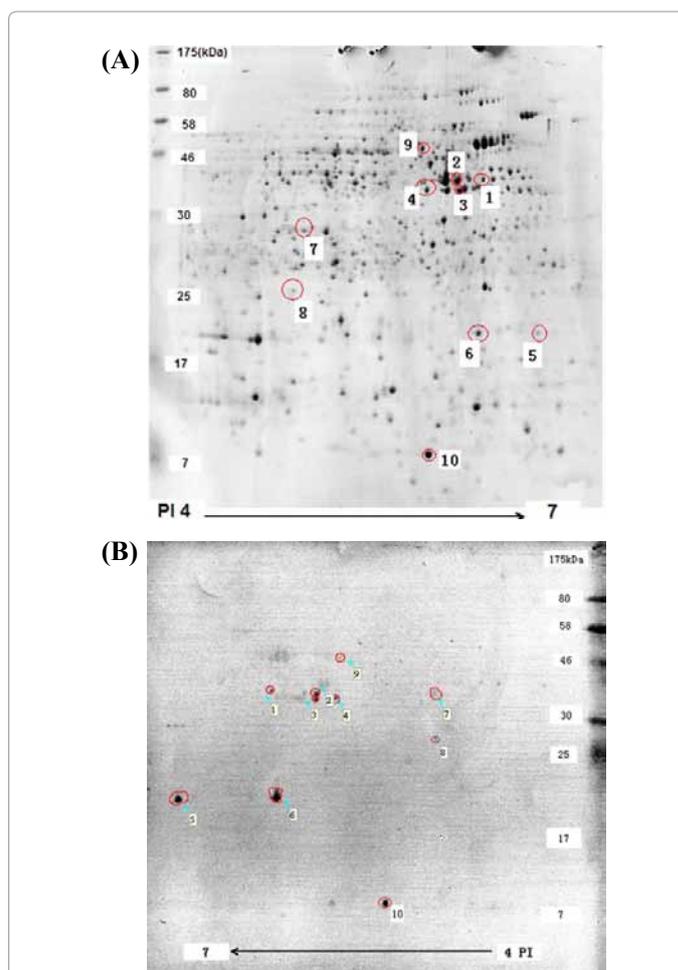
Total membrane proteins of *B. melitensis* M5 strain were separated by 2-DE. Three technical replicates of 2-DE gels for the membrane proteins were run. A total of 800  $\mu$ g of extracted proteins were loaded into the IPG strips then to separate in 12.5% SDS-PAGE. After being stained with colloidal Coomassie Brilliant Blue G-250, over 600 spots could be detected on the gels within the pI range 4–7 and the MW range 7 – 175 kDa (Figure 1).

### Immunoreactive proteins

Western blotting analysis of 2-D gel using antisera from humans naturally infected by *B. melitensis* as primary antibody and rabbit anti-goats IgG as secondary antibody revealed a total of 10 immunoreactive protein dots (Figure 2b), consistent with our observations on the duplicated 2-D gel (Figure 2a). The corresponding stained dots in



**Figure 1:** 2-DE profile of membrane proteins from Chinese *Brucella* vaccine strain M5. A total of 800 $\mu$ g membrane proteins from Chinese *Brucella* vaccine strain M5 were separated by two-dimensional electrophoresis (IPG 4–7, MW7–175kDa) Stained with CBB G250, over 600 protein dots were detected.



**Figure 2:** 2-D Western blot analysis of the total membrane proteome of Chinese *Brucella* vaccine strain M5. The 2-D gel was electroblotted onto PVDF membrane (20V, 1h) and western blotting analysis was performed with antisera from *Brucella*-infected goats. A total of ten immunodominant protein dots were recognized by antiserum and labeled with the Arabic numbers on the 2-D gel (Figure 2-A) and PVDF membrane (Figure 2-B), respectively.

the 2-D gel were excised, digested and analyzed by LC-MS/MS. 10 protein dots could be identified which corresponded to 9 different gene products (Table 1).

### Subcellular localization, signal peptides and other information

PSORTb analysis predicted that 5 of the 9 immunogenic proteins

are localized in cytoplasm, 3 proteins on outer membrane and 1 protein in the periplasm of the organism. According to Signal P analysis, only 1 of the 9 immunogenic proteins has a signal peptide sequence. Searching for these proteins in <http://www.expasy.ch/> showed that they participate in inner- and outer-membrane transport (3/9), protein biosynthesis (3/9), substance metabolism (2/9) and bacterial virulence system (1/9) (Table 1).

Position in gel	Protein Name <sup>a</sup>	Genename /Site number	Access Number	pl / M.W	Metabolism <sup>b</sup> pathway	Signal Peptide <sup>c</sup>	Subcellular <sup>d</sup> localization
Point 1	maltose binding protein	BMEI1716	NP_540633.1	5.09/46228	transport	no	P
Point 3	isovalery-CoA dehydrogenase	ivd	NP_540840.1	5.36/41428	metabolic process	no	C
Point 5	25kDa-outer membrane immunogenic protein precursor	BMEI1829	NP_540746.1	4.78/24571	transport	yes	OM
Point 6	31kDa-outer membrane immunogenic protein precursor	omp31	NP_541822.1	5.21/23258	ion transport	no	OM
Point 7	glycerol trinitrate reductase	BMEI0394	NP_541372.1	5.80/39981	metabolic process	no	C
Point 8	succinyl-CoA synthetase subunit alpha	BMEI0139	NP_539057.1	5.81/31214	tricarboxylic acid cycle	no	C
Point 10	peptidyl-prolyl cis-trans isomerase A	PPI	NP_539805.1	6.08/19145	protein processing	no	OM
Point 9	S-adenosyl-L-homocysteine hydrolase	ahcY	NP_540946.1	5.30/52430	Amino-acid biosynthesis	no	C
Point 2	Protein translation elongation factor EF-TU	tufA/tufB	AAL51923.1	5.60/44313	protein biosynthesis	no	C

The immunodominant protein dots were subjected to trypsin digestion and LC-MS/MS identification. The results were subjected to bioinformatics retrieval, signal peptide prediction, subcellular localizations confirmation and searching for metabolism pathways.

a. The Arabic numbers are concord with the gel and PVDF membrane.

b. The metabolism pathways of identified proteins were searched in the Web of <http://www.kegg.com/>.

c. Signal peptide prediction was accomplished through SignalP 3.0 software (<http://www.cbs.dtu.dk/services/SignalP/>). "+", with signaling peptide; "-", without signaling peptide.

d. Subcellular localizations were predicted by PSORTb v2.0 software (<http://www.psort.org/>). C, Cytoplasmic; P, Periplasmic; OM, Outer Membrane

**Table 1:** The results for LC-MS/MS identification and information retrieval on immunoreactive proteins.

Primers number	Primer sequences(5'-3')	protein names and position in gel	Length of PCRproducts (bp)	PCR conditions (°C/s)		PCR results of four strains of Brucella			
				Denaturation	Annealing	M5	16M	544A	S19
1	<i>catatgatgggaattgcccggttac</i> <i>ctcagatcaaaaggtcggcgctcttc</i>	maltose binding protein (point 1)	1272	94/30	60/30	+	+	+	+
				72/60					
2	<i>catatgatgaacttcggcttggcgag</i> <i>ctcagatcaacgggtctcctgaaagag</i>	isovalery-CoA dehydrogenase (point 3)	1149	94/30	58/30	+	+	+	+
				72/60					
3	<i>catatgatgaagctcaaggctcttc</i> <i>ctcagatcagaactgtaagcgacac</i>	25kDa-outer membrane immunogenic protein precursor (point 5)	687	94/30	55/30	+	+	+	+
				72/60					
4	<i>catatggtggtgtttctgaacctccg</i> <i>ctcagatgtagaactgtagttagaccgacg</i>	31kDa-outer membrane immunogenic protein precursor (point 6)	660	94/30	58/30	+	+	-	-
				72/60					
5	<i>gagctcatggcctcattgttcgacccattac</i> <i>ctcagatcatgccaagaacgggataatc</i>	glycerol trinitrate reductase (point7)	1116	94/30	58/30	+	+	+	+
				72/60					
6	<i>gagctc atgcaattttgccttcag</i> <i>ctcagatgaataacctgtagtggcacttg</i>	succinyl-CoA synthetase subunitalpha (point 8)	903	94/30	58/30	+	+	+	+
				72/60					
7	<i>catatggtgcaaatcaacagagagggc</i> <i>ctcagatcagatgctggcgagac</i>	peptidyl-prolyl cis-trans isomeraseA (point 10)	531	94/30	57/30	+	+	+	+
				72/60					
8	<i>catatgatgtccattctcgtcaacaag</i> <i>ctcagatcagcccttgaggactcaac</i>	S-adenosyl-L-homocysteine hydrolase (point 9)	1446	94/30	58/30	+	+	+	+
				72/60					
9	<i>catatgatggcaagagtaagttgaacgtac</i> <i>ctcagat tactcgtgatcgacgagacg</i>	Protein translation elongation factor EF-TU (point 2)	1176	94/30	58/30	+	+	+	+
				72/60					

The primers for the genes of the identified proteins were designed and amplified by PCR in four *Brucella* strains. The optimized PCR conditions and the length of PCR products were determined. The results of the PCR amplification in four different *Brucella* strains were shown. "+", Corresponding sequence have been able to amplify; "-", Corresponding sequence not been able to amplify

**Table 2:** Primers for genes of the identified proteins and PCR results.

### PCR detection

PCR primers, amplification condition and product length are shown in Table 2. The PCR results confirmed that the eight primer pairs employed were able to amplify genomic sequences from *B. melitensis* 16M strain and M5 strain, *B. abortus* 544A strain and S19 strain. The primers for the *Omp31* gene did not yield products in 544A and S19. (Figure 3A). The multiple sequence alignment of the DNA sequences is shown in Figure 3B.

### Antigenicity analysis

Combining the results according to the methods mentioned above, the antigenicity for the immunoreactive proteins identified in *B. melitensis* M5 vaccine strain membrane proteins was predicted. The results demonstrated that the B-cell epitopes localized in or nearby these proteins N-terminus, and there were some centers of  $\alpha$ -helix,  $\beta$ -sheet and flexible regions in N-terminal (the data and figures are not shown). These results are helpful for identification of potential antigen candidates for *Brucella* subunit vaccines development.

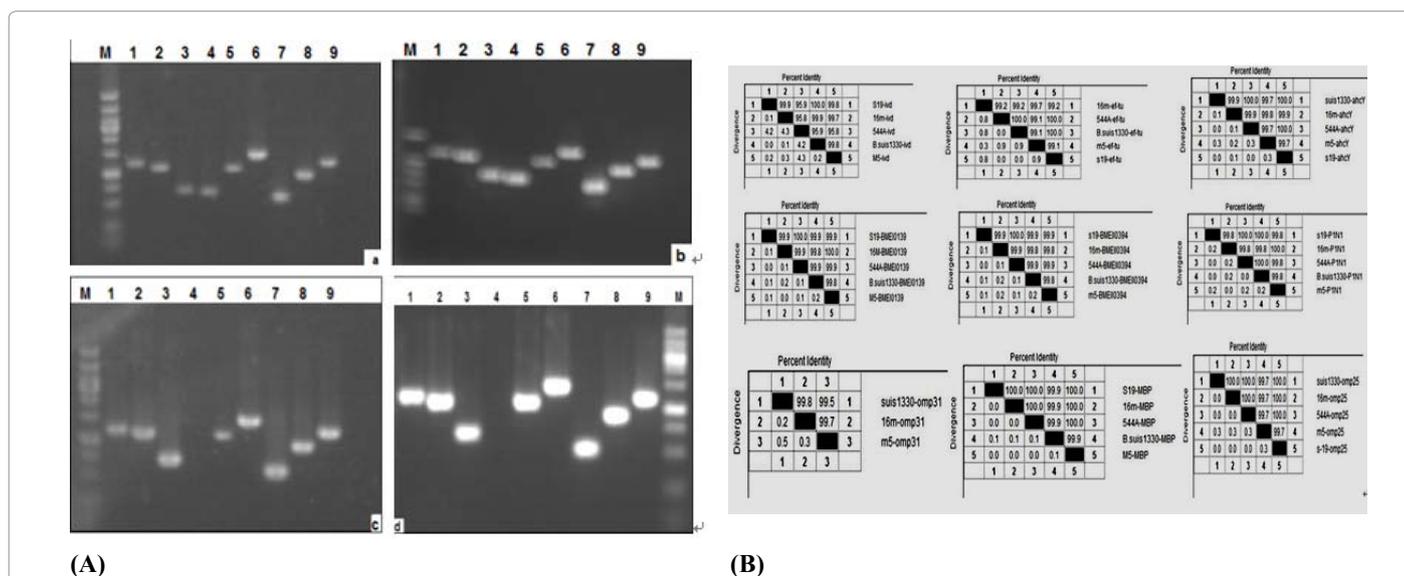
### Discussion

The development of new-generation vaccine systems to prevent brucellosis is needed to overcome the disadvantages of the live vaccines currently used. An attractive approach is the development of subunit vaccines. Several antigens that induce protective responses have been localized in the outer membrane of *Brucella* cells. Bacterial surface antigens are prime candidates for vaccines as they represent the initial point of contact between the pathogen and their host [8,10,44,45]. In the present study, the immunogenic proteins from *B. melitensis* vaccine strain M5 membrane proteins were identified by an immunoproteomics strategy. However, producing high quality two-dimensional electrophoretic gels of bacterial cell membrane proteins is challenging because of non-membrane protein impurities within

cell membrane preparations and the intractability of membrane proteins for solubilization. In the current study, a sample preparation method combining carbonate extraction, novel zwitter ionic detergents and surfactant-free organic solvent-assisted solubilization was chosen for the isolating process of *Brucella* cell membrane. In some previous studies, the combination of carbonate extraction and strong zwitterionic detergents-assisted solubilization for membrane proteins has confirmed that it is a useful strategy for separating membrane proteins with 2-DE [46,47]. As shown in Figure 1, more than 600 protein spots were isolated with a highly qualitative 2-DE profile. For all this, the proportion of membrane protein was still lower, out of 9 immunodominant proteins probed with antisera from *Brucella*-infected goats, 5 were *Brucella* cell membrane constituents based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Table 1). Cell membranes containing integral membrane proteins, and some *N*-acyl diglyceride attached lipoproteins, remain insoluble in the carbonate solution. Integral membrane proteins belong to the category of proteins which have been difficult to characterize *via* a conventional 2-DE approach, primarily because of their tendency to aggregate and precipitate during the separation step of the first dimension, and secondarily, due to their typically lower abundance in the cell compared to soluble proteins.

Subcellular fractionation and enrichment techniques can overcome many of the challenges of low abundance, but solubility issues remain a challenge [48,49].

In this study, we have presented the immune proteomic analysis of the *B. melitensis* M5 strain membrane proteins. This analysis revealed a few proteins already identified in *Brucella* membrane, such as OMP25, OMP31. But also a great number of other proteins that are yet to be annotated were also identified in the cell membrane. These include proteins involved in protein translation, energy production, and reduction/oxidation reactions (*i.e.*, maltose binding protein,



**Figure 3** PCR amplification and sequence distance analysis on the genes encoding identified proteins. **A:** The immunodominant proteins identified by LC-MS/MS were amplified by PCR from *Brucella melitensis* 16M strain (a), M5 strain (b); *Brucella abortus* 544A strain (c) and S19 strain (d). M, DNA Marker; 1, maltose binding protein; 2, isovalery-CoA dehydrogenase; 3, 25kDa-outer membrane immunogenic protein precursor; 4, 31kDa-outer membrane immunogenic protein precursor; 5, glycerol trinitrate reductase; 6, succinyl-CoA synthetase subunit alpha; 7, peptidyl-prolyl cis-trans isomerase A; 8, S-adenosyl-L-homocysteine hydrolase; 9, Protein translation elongation factor EF-TU. **B:** All the PCR products of immunodominant proteins were inserted into PMD-18T vector. Following sequencing, Sequence Distances among these sequences were analyzed through DNAMAN, and the gene homologies ranging from 95%-99% among five *Brucella* strains.

isovaleryl-CoA dehydrogenase, glycerol trinitrate reductase, succinyl-CoA synthetase subunit alpha, peptidyl-prolyl cis-trans isomerase A, S-adenosyl-L-homocysteine hydrolase, Protein translation elongation factor EF-TU). Recent membrane proteomic work on other prokaryotes has resulted in similar findings [50]. In our work, the 9 immunodominant proteins were divided into three groups. The first group comprised 2 *Brucella* immunogenic proteins (OMP25 and Omp31) that have been previously identified to be putatively immunogenic in *Brucella* spp.. Those are the most representative third group outer membrane proteins (25-34 kDa) of *Brucella* [51] and their immunogenicity and protective ability have been confirmed in previous investigations [52,53]. The second group of immunodominant proteins included EF-TU (protein dot 2), Maltose-binding protein (MBP, protein dot 1), peptidyl-prolyl-cis-trans isomerase (PPIases, protein dot 10) have already been shown to be antigenic in certain pathogenic bacteria, but not in *B. melitensis* before. Membrane-linked protein translation elongation factor EF-TU is highly expressed and has multiple subcellular localizations. It has been found to be antigenic in many bacteria, such as *Helicobacter Pylori* [54], *Shigella flexneri* [22], *S. epidermidis* [55], *Leptospira* spp [56] and *Chlamydia pneumoniae* [23] through immunoproteomics. In *E.coli*, EF-Tu has been found to interact with an inner membrane protein MdtE, an outer membrane protein OmpF and a transmembrane protein OmpA in the outer membrane. These four proteins form a multi-protein complex, naming EF-Tu multiprotein complex. EF-Tu directly binds to OmpA, indicating that EF-Tu plays an important role in the events [57]. Maltose-binding protein (MBP, protein dot 1) is distributed in the periplasmic space and proved to elicit cellular immune response [43]. A recent study indicated that MBP can promote the maturation of DC [58]. The PPIases, as a virulence factor was reported for *Legionella pneumophila* [59,60] and as an immunoreactive protein in *Bordetella pertussis* [60]. In the third group, immunodominant proteins were those identified as immunogenic candidates for the first time in this study. They are isovaleryl coenzyme A dehydrogenase (protein dot 3), nitroglycerin reductase (protein dot 7), succinyl-CoA synthetase subunit (protein dot 8) and S-adenosine-L-homocysteine hydrolase (protein dot 9, ahcY). Nitroglycerin reductase is an important enzyme in plant nitrogen metabolism. However, we found that it has been important component of *Brucella* two-component virulence system by searching in the KEGG metabolism pathway in present study. The correlation between Nitroglycerin reductase and the virulence of *Brucella* remains to be elucidated. The ahcY plays a pivotal role in protein methylation and has become one of the important targets for the development of broad-spectrum antiviral drugs [61].

In our study, the Omp31 gene has been amplified in *B. melitensis* (Figure 3Aa and 3Ab) but not in *B. abortus* (Figure 3Ac and 3Ad). A BLAST search revealed that the Omp31 gene from *B. melitensis* was 126 bp shorter than that from *B. abortus*. The Omp31 is typical and putative protein presenting in 6 *Brucella* species. Previous studies confirmed that the Omp31 family have a different distribution according to host preference they exhibit [62]. In addition, 7 immunodominant proteins mentioned above are highly conserved among *Brucella* species. Therefore, it is rational to suggest that these proteins may be promising vaccine candidates. The identification of new immune reactive proteins from the *Brucella* Chinese vaccine strain M5 membrane proteins adds to the list of potential antigens that can be used in the development of new vaccines. The protective capacities of the immunogenic proteins identified either alone or in different combinations remain to be determined in further studies. In the future we will focus on expressing candidate antigenic proteins and testing their immunogenicity.

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