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Immunoproteomic analysis of *Brucella melitensis* and identification of a new immunogenic candidate protein for the development of brucellosis subunit vaccine

Yanling Yang[a,b,1], Lin Wang[d,1], Jigang Yin[c,1], Xinglong Wang[a,c,*], Shipeng Cheng[b], Xulong Lang[a], Xiuran Wang[a], Hailong Qu[a], Chunhui Sun[a], Jinglong Wang[a], Rui Zhang[a]

[a] *The Eleventh Institute of the Academy of Military Medical Sciences of PLA, Changchun 130122, China*
[b] *Institution of Special Wild Economic Animal and Plant Science, CAAS, Jilin 132109, China*
[c] *Key Laboratory of Zoonosis Research, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, China*
[d] *Department of Pharmacology, School of Pharmacy, Second Military Medical University, Shanghai 200433, China*

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ABSTRACT

In order to screen immunogenic candidate antigens for the development of a brucellosis subunit vaccine, an immunoproteomic assay was used to identify immunogenic proteins from *Brucella melitensis* 16 M soluble proteins. In this study, a total of 56 immunodominant proteins were identified from the two-dimensional electrophoresis immunoblot profiles by liquid chromatography tandem mass spectrometry (LC–MS/MS). Two proteins of interest, riboflavin synthase alpha chain (RS-α) and Loraine synthase (LS-2), which are both involved in riboflavin synthesis, were detected by two-dimensional immunoblots using antisera obtained from *Brucella*-infected human and goats. LS-2, however, is an already well-known vaccine candidate. Therefore, we focussed our studies on the novel vaccine candidate RS-α. *B. melitensis* RS-α and LS-2 were then expressed in *Escherichia coli* as fusion proteins with His tag. The humoral and cellular immune responses to the recombinant (r)RS-α was characterized. In response to in vitro stimulation by rRS-α, splenocytes from mice vaccinated with rRS-α were able to produce γ-interferon (IFN-γ) and interleukin (IL)-2 but not interleukin (IL)-4 and interleukin (IL)-10. Furthermore, rRS-α or rLS-2-vaccinated mice were partially protected against *B. melitensis* infection. Our results suggested that we have developed a high-throughout, accurate, rapid and highly efficient method for the identification of candidate antigens by a combination of immunoproteomics with immunisation and bacterial challenge and rRs-α could be a useful candidate for the development of subunit vaccines against *B. melitensis*.

1. Introduction

Brucella melitensis is a zoonotic Gram-negative pathogen that causes abortion and infertility in ruminants, and undulant fever characterized by malaise, aches, and fevers in humans (Seleem et al., 2010). In regions with high prevalence of the disease, the only way of controlling of the disease is by vaccination of all susceptible hosts and elimination of infected animals (Kakoma et al., 2007). Because of the serious economic and medical consequences of brucellosis, efforts have been made through the use of vaccines to prevent the infection. All commercially available brucellosis vaccines are based on live, attenuated strains of *Brucella*. Although effective, these vaccines have disadvantages: they can be infectious for humans; they can interfere with diagnosis; they may result in abortions when administered to pregnant animals; and the vaccine strain can spread in the region (Ashford et al., 2004; Nicoletti, 2010). Currently, no vaccine against human brucellosis is available (Thakur et al., 2002). Therefore, improved vaccines that combine safety and efficacy to all species at risk need to be designed. Within the past few years, efforts has been made to develop a vaccine without these drawbacks (Cassataro et al., 2007; Delpino et al., 2007; Luo et al., 2006a; Pasquevich et al., 2009). Subunit vaccines, like recombinant proteins, are promising vaccine candidates, because they can be produced at high yield and purity and can be manipulated to maximize desirable activities and minimize undesirable ones. Moreover, they are safer for manipulators, well defined, not infectious, and cannot revert to a virulent strain. Numerous *Brucella* cell surface and intracellular components have been assessed as protective antigens. Significant activity has been identified for only a few antigens: the L7/L12 ribosomal protein (Mallick et al., 2007),

* Corresponding author at: The Eleventh Institute of the Academy of Military Medical Sciences of PLA, Changchun 130122, China. Tel.: +86 431 86985951; fax: +86 431 86979779.
E-mail addresses: yangyanling12280@sina.com (Y. Yang), musics@sina.com (L. Wang), yinjg0924@sina.com (J. Yin), wangxl-2006@163.com (X. Wang).
[1] These authors contributed equally to this work.

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the Cu–Zn superoxide dismutase (Tabatabai and Pugh, 1994), a 22.9-kDa protein (Cespedes et al., 2000), the cytoplasmic protein p39 (Al-Mariri et al., 2001) and *Brucella* lumazine synthase (BLS) (Velikovsky et al., 2002). However, they tend to be poorly immunogenic and protective ability *in vivo*, and require identification of more effective antigens or co-administration of better adjuvants that enhance the immune response against recombinant proteins.

A new revolution in vaccine design has emerged from the use of post-genomic technologies (Khan et al., 2006; Serruto and Rappuoli, 2006). Much of information about immunogenic components can be derived from proteomics coupled to Western blotting, namely immunoproteomics which has been successfully applied for the discovery of antigens from various bacterial pathogens (Altindis et al., 2009). Recently, immunoproteomics has considerably facilitated the identification of immunogenic proteins in *Brucellae* (Connolly et al., 2006; Teixeira-Gomes et al., 1997). Although many immunogens can be identified, immunogens do not always equate to protective antigens. Evaluation of the immunogenicity and protective ability of all immunogens is likely to be helpful for developing effective vaccines.

Immunity against *Brucella* requires cell-mediated mechanisms (Araya et al., 1989; Araya and Winter, 1990; Cheers, 1984). In particular, Th1 immune responses characterized by production of gamma interferon (IFN-γ) are associated with protective immunity to *Brucella* (Eze et al., 2000; Murphy et al., 2001). These responses are best stimulated by live vaccines or potentially by multiple injections of appropriate protective antigens in the presence of adjuvants which favor cell-mediated immune mechanisms. The difficulty is that few effective candidate antigens have yet been identified.

In the present study, this paper describes the immunoproteomic approach for the identification of immunogenic proteins from the total soluble proteomes of *B. melitensis* 16 M strain. Two candidate immunogenic proteins of interest, riboflavin synthase alpha chain (RS-α) and lumazine synthase 2 (LS-2, ribH2), were recognised by using antiserum from *Brucella*-infected goats and patients. In preliminary enzymatic studies, the enzyme lumazine synthase (LS) was shown to catalyze the formation of 6,7-dimethyl-8-ribityllumazine, the penultimate step in the biosynthesis of riboflavin (Zylberman et al., 2006). A phylogenetic analysis on eubacterial, fungal and plant LSs allowed us to classify them into two categories: Type-I LSs (pentameric or icosahedral) and Type-II LSs (decameric). *Brucella* codes both a Type-I and a Type-II LS called RibH1 and RibH2, respectively. LS activity *in vivo* has confirmed that RibH2 and not RibH1 is essential for intracellular survival (Bonomi et al., 2010). In previous reports, however, lumazine synthase (LS-2) is an already well-characterized vaccine candidate, mice injected with plasmid DNA encoding LS induces a Th1-specific immune response and protection against *B. abortus* challenge (Velikovsky et al., 2002). Although the reports about the Riboflavin synthase alpha chain (RS-α, EC 2.5.1.9) is less, the enzyme is shown to catalyze the last step in the biosynthesis of riboflavin, catalyzing 6,7-dimethyl-8-(1-D-ribityl) lumazine to yield riboflavin, it would have been of greater scientific interest to study Rsα as a novel vaccine candidate instead. Therefore, the immunogenicity and protective efficacy of rRS-α was further evaluated in mouse model.

2. Materials and methods

2.1. Bacterial strains and culture conditions

B. melitensis 16 M, a virulent biotype I reference strain, was used for immunoproteomics research and infection experiments and *B. melitensis* Rev.1, a vaccine strain, was used as a control for immunising mice. Both of them were obtained from the National Institute for Control of Veterinary Bioproducts and Pharmaceuticals (NICVBP, China).

B. melitensis 16 M and Rev.1 stock samples were inoculated in tryptic soy broth (TSB; FLUKA, USA) at 37 °C with vigorous shaking (250 rpm) for 48 h to OD_{600} of 2.8. The exact dose counts were visually assessed on tryptic soy agar (TSA; FLUKA, USA) plates. Bacteria were collected by centrifugation and resuspended in sterile phosphate-buffered saline (PBS).

2.2. Animals

The 4–6-week-old female SPF (specific pathogen-free) BALB/c mice were purchased from the animal centre of Jilin University (Changchun, China). Mice were bred in the animal facilities with filtered inflow air in a restricted-access room and under pathogen limited conditions. Mice were acclimatised for a minimum of one week prior to experimentation and water and commercial food were provided *ad libitum*. All animal experiments were approved by the Center of Laboratory Animals in Jilin Province, China.

2.3. Extraction of bacterial proteins

Protein extraction was performed as previously reported by Henningsen et al. (2002). Briefly, bacteria were collected at 4 °C, 6000 × *g* for 10 min, washed four times with ice-cold PBS, resuspended in 50 mM Tris–HCl (pH 7.5) to reach a final volume of 5 mL. Cells were sonicated for 10 min on ice using a Sonifier 750 (Branson Ultrasonics Corp., Danbury, CT, USA) with the following parameters: 2 s of sonication with a 2 s interval, 25% highest power, and centrifuged at 4 °C, 2000 × *g* for 10 min. The supernatant was collected and mixed with 10 volumes of ice cold 0.1 M Na_2CO_3 (pH 11.0). The mixture was incubated in an ice bath with constant gentle agitation 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–HCl (pH 7.5) and centrifuged at 4 °C, 100 000 × *g* for 1 h. The supernatant was discarded, and the pellet was lysed in 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 and the protein concentration measured using the PlusOne 2-D Quant Kit (GE Healthcare, USA). Aliquots of 800 μg supernatant were subjected to SDS-PAGE analysis or kept at −70 °C.

2.4. Two-dimensional electrophoresis (2-DE)

2.4.1. Isoelectric focusing (IEF)

2-DE was performed as described by Görg with modifications (Görg et al., 2004). Briefly, 18 cm immobilised pH gradient (IPG) strips (pH range, 4–7) were rehydrated at room temperature in a passive rehydration tray for 20 h with 350 μL sample containing 800 μg total protein. Isoelectric focusing (IEF) was conducted at 20 °C for 16 h. The parameters used for IEF were: 50 V for 2 h; 500 V for 1 h; 1000 V for 1 h; 3000 V for 3 h followed by a linear increase to 8000 V for 3 h. The final phase of 8000 V was terminated after 48,000 V h.

2.4.2. SDS-PAGE

After IEF, strips were equilibrated in 5 mL equilibration buffer I (50 mM Tris–HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, trace bromphenol, 50 mg DTT) on a horizontal shaker for 15 min, followed by equilibration in 5 mL equilibration buffer II (50 mM Tris–HCl [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, USA) were loaded into homogeneous 12.5% polyacrylamide gels