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Proteomics-based identification of immunodominant proteins of Brucellae using sera from infected hosts points towards enhanced pathogen survival during the infection

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ABSTRACT

Brucella (*B.*) species lack classical virulence factors, but escape effectively the immune response of the host. The species *Brucella abortus* and *Brucella melitensis* infect predominantly cattle and small ruminants such as sheep or goats, respectively, but account also for most human cases. These two species share remarkably similar genomes but different proteomes have been demonstrated. This might be one of the reasons for their host specificity. A comprehensive identification of immunodominant proteins of these two species using antibodies present in the serum of naturally infected ruminants might provide insight on the mechanism of their infection in different hosts. In the present study, whole-cell protein extracts of *B. abortus* and *B. melitensis* were separated using SDS–PAGE and western blotting was performed using field sera from cows, buffaloes, sheep and goats. Protein bands that matched with western blot signals were excised, digested with trypsin and subjected to protein identification using MALDI-TOF MS. Identified proteins included heat shock proteins, enzymes, binding proteins and hypothetical proteins. Antibodies against the same set of antigen were found for all species investigated, except for superoxide dismutase of *B. melitensis* for which antibodies were demonstrated only in sheep serum. Brucellae appear to express these proteins mainly for their survival in the host system during infection.

1. Introduction

Brucella (*B.*) is a facultative intracellular pathogen that currently includes 11 generally accepted nomo-species which were named based on their antigenic and biochemical characteristics and primary host species specificity [1,2]. *Brucella* species lack classical virulence factors like exotoxins, flagella, pathogenicity islands, or genes for type I, II and III secretion systems responsible for host–bacteria interaction [3–5]. It has been demonstrated that Brucellae are capable of arranging extensive reversible modifications in their cell envelope as an adaptation to changing microenvironment within the host cells and induce modulations in host immune response to enhance their intracellular survival [6]. Despite the high genomic similarity among the *Brucella* species [7], it has been demonstrated that the same species evoke different immune responses in experimental and natural hosts [8]. The two well-known human pathogens, *Brucella abortus* (preferred host: cows and buffalos) and *Brucella melitensis* (goats and sheep) share remarkably similar genomes [7,9,10] but display different protein expression profiles [11]. The presence of specific antibodies in the host system might reflect the status of immune response and the actual degree of antigen exposure during the infection. Hence, it is expected that their natural hosts might develop antibodies against proteins related to host specificity. However, earlier studies aimed towards identification of bacterial or host-species specific immunodominant proteins appear to be inconclusive due to a limited number of host samples, use of hyperimmune serum of experimentally infected non-natural hosts or use of *Brucella* reference strains which have a museum-like quality and do not represent current field strains [12–16]. Therefore, the present study aimed at comprehensive identification of the immunodominant proteins from two different field strains of *B. abortus* and *B. melitensis* and sera collected from their naturally preferred infected hosts, i.e., bovines (cow and buffalo) and small ruminants (goat and sheep), respectively.

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2. Materials and methods

2.1. Choice of anti-sera

A total (n = 24) of three animals naturally infected with *Brucella* (positive) and three non-infected (negative) sera of each host species i.e., cattle, buffaloes, sheep and goats were analyzed in this study. The negative or positive tests status of serum samples was characterized using the recommended tests [17], Rose Bengal Test (RBT: any degree of agglutination), Complement Fixation Test (CFT: 50% or less hemolysis at a dilution of 1:4 or greater i.e., $\geqslant$20 IU/mL) and as per the manufacturer's recommended cut-off value of ELISA using *Brucella* S-LPS as antigen (IDEXX Brucellosis serum X2 Ab test, Montpellier SAS, France).

All seropositive and buffalo seronegative samples were collected during routine diagnostics as approved by the ethical committee at the office of dean, Faculty of Veterinary Medicine, Benha University, Ministry of Higher Education, Egypt. The remaining seronegative samples were from the collection of Friedrich-Loeffler-Institut (FLI), Jena, Germany.

2.2. Choice of Brucella species

The bacterial strains used in this study were taken from the culture collection of the Institute for Bacterial Infections and Zoonoses (IBIZ); Friedrich-Loeffler-Institut (FLI); Jena; Germany. The *B. abortus* field strain was originally isolated from cattle in Turkey while the *B. melitensis* field strain was isolated from sheep in China. Species identification was carried out based on CO_2 requirement, H_2S production, growth in the presence of dyes, reaction with mono-specific sera, and phage lyses as described [17]. Molecular species identification was confirmed by real-time PCR as previously described [18].

2.3. Protein extraction

The whole-cell protein extraction was carried out as described [19] with minor modifications. In brief, strains were cultured for 48 h in Tryptic Soy Broth (TSB) at 37 °C with shaking, harvested during the stationary growth phase by centrifugation and washed twice with phosphate buffer saline. The cells were reconstituted in 80% ethanol and centrifuged. Then, the cell pellet was air dried and reconstituted in lysis buffer (20 mM HEPES, pH 7.4 containing 1 mM EDTA, 0.5% benzonase, 0.5% protease inhibitors, 10% glycerol and 1% Triton X100), sonicated on ice for 1 min (duty cycle: 1.0, amplitude: 100%, UP100H; Hielscher Ultrasound Technology, Teltow, Germany), centrifuged at 11,290*g* for 10 min at 4 °C and the supernatant was collected and stored at −20 °C until further use.

2.4. Western blotting

Western blotting was carried out as described [20] with minor modifications. All of the secondary antibodies were procured from Biomol-Rockland, Hamburg, Germany. 100 μg of total protein lysate was separated using an SDS–polyacrylamide gel and blotted onto a nitrocellulose membrane (Bio-Rad, Munich, Germany). Bovine sera (1:200 diluted) and small ruminants sera (1:500 diluted) were used as primary antibody source while 1:1000 diluted anti-bovine IgG (H&L) (Chicken) peroxidase-conjugated, anti-sheep IgG (H&L) (Donkey) peroxidase-conjugated and anti-goat IgG (H&L) (Chicken) peroxidase-conjugated antibody served as secondary antibody source. The detection of signals was carried out using the TMB kit™ (3,3′,5,5′-tetramethylbenzidine liquid substrate, Sigma–Aldrich, Steinheim, Germany).

2.5. Protein identification

The protein bands that corresponded to the western blot signals were excised from the SDS–PAGE gels and digested with trypsin. The gel pieces were distained by shacking (1200 rpm, Thermomixer Pro, Cell Media, Gutenborn, Germany) at RT for 30 min in three subsequent steps, each by adding 100 μl of the following solutions: (1) 200 mM ammonium bicarbonate (Sigma–Aldrich, Steinheim, Germany) (2) 200 mM ammonium bicarbonate in 50% acetonitrile (Sigma–Aldrich, Steinheim, Germany) and (3) 20 mM ammonium bicarbonate in 5% acetonitrile. Following this, 50 μl of acetonitrile was added to each tube, incubated at RT for 5 min, the supernatant was discarded and the remaining gel pieces were dried using a vacuum centrifuge (UniVapo 100H, Uniequip, Martinsried, Germany). The gel pieces were then rehydrated with 20 μl trypsin solution (0.01 μg/μl) (Promega, Mannheim, Germany) in 20 mM ammonium bicarbonate in 5% acetonitrile and incubated at 37 °C for 12 h. Subsequently, the resulting peptides were extracted using 25 μl of acetonitrile. The extraction was repeated 3 times, the resulting fractions were pooled and dried completely using vacuum centrifuge. The precipitates were then reconstituted and spotted onto MALDI target plate using HCCA (α-Cyano-4-hydroxycinnamic acid, Sigma–Aldrich, Steinheim, Germany) matrix as described [19]. The MALDI TOF MS/MS measurement was carried out using Ultraflex II TOF/TOF (Bruker Daltonics, Bremen, Germany). Afterwards, a database search for protein identification was performed using MS/MS ion search (MASCOT, www.matrixscience.com) against all entries of NCBInr (GenBank) as described [19]. The protein identification was considered valid if matched more than 2 peptides and the MOWSE score was significant ($p < 0.05$).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [21] via the PRIDE partner repository with the dataset identifier PXD001270 and DOI http://dx.doi.org/10.6019/PXD001270.

3. Results

3.1. Antigenic proteins of B. abortus and B. melitensis

SDS–PAGE separation of whole-cell protein extracts of *B. abortus* and *B. melitensis* are shown in Fig. 1. The protein patterns obtained with both isolates were comparable, however differences in terms of intensities were observed. A number of bands were distinct in both isolates, e.g., in the range between 50 kDa and 70 kDa a total of 5 bands were observed in the case of *B. abortus* whereas *B. melitensis* displayed only 3 bands in the same region.

3.2. Western blotting

Western blotting revealed signals for at least 22 immunoreactive bands (Fig. 2). *B. abortus* and *B. melitensis* appear to differ in terms of detectable signals when using sera from bovines or small ruminants, respectively. As shown in Fig. 2, the sera used in this study contained antibodies against lower molecular weight proteins in the range up to 30 kDa (12 bands), and one common band in the 60 kDa region. These protein bands were consistently detected in the soluble protein fractions of *B. abortus* and *B. melitensis* with all sera regardless of whether they were of bovine, ovine or caprine origin. At least four clear signals were observed in the range of 70–100 kDa in the case of *B. abortus*. All host serum samples displayed no signals specific for host preference except two protein bands from *B. melitensis* that were specific for sheep (Fig. 2, M09 and M10). As expected no signals were detected when negative sera were used as the primary source of antibodies.