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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.

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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 11,290g 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-hydroxy-cinnamic 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.matrix-science.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.
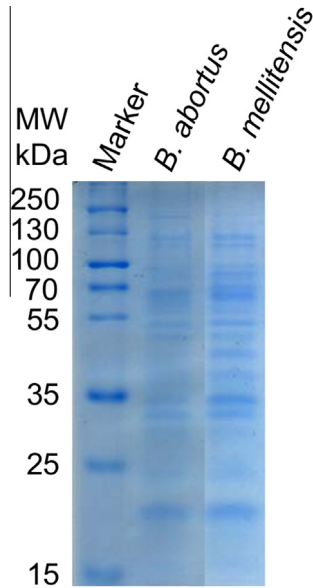
Fig. 1. SDS–PAGE of whole-cell protein extracts from *Brucella abortus* and *Brucella melitensis*.

3.3. Protein identification

Proteins from 18 bands (8 for *B. abortus* and 10 for *B. melitensis*) were identified. All identified proteins are immunoreactive with all sera from cattle, buffaloes, sheep and goats except superoxide dismutase from *B. melitensis* specific for sheep only (Table 1). The identified proteins were mainly enzymes (superoxide dismutase, catalase, transaldolase, glutamine amidotransferase and fumarylacetoacetate hydrolase domain-containing protein 2), heat shock proteins (molecular chaperone DnaK and GroEL), or binding proteins (ribose transport system substrate-binding protein, sn-glycerol-3-phosphate-binding periplasmic protein ugpB, amino acid ABC transporter substrate-binding protein and D-ribose-binding periplasmic protein precursor).

4. Discussion

Earlier attempts to identify *Brucella* immunodominant proteins suffered from shortcomings: a limited number of host samples, use of unnatural hosts for experimental infection and use of laboratory-adapted type strains [12–16]. It can be supposed that the results of these investigations are biased or distorted in regard to validity for the actual situation in the field. It is astonishing that only two proteins, 26-kDa cytosoluble protein (Bp26) and periplasmic serine protease (HtrA/DegP) are immunoreactive in human and goats [8], but the expression of antibodies against Bp26 in goats and sheep appears to be influenced by the strain of *B. abortus* and *B. melitensis* itself rather than bacterial species [22]. These finding underscores the need for a comprehensive study using current field strains and sera from naturally infected host animals to define the immunodominant proteins in the zoonotic *B. abortus* and *B. melitensis*. Both species have remarkably similar genomes [9,10] and intracellular life style, but display different proteomes [11] and have a clear specificity towards their primary hosts [23].

The present study investigates sera from naturally infected animals which were tested negative for *Yersinia* antibodies to avoid any misidentification or cross-reactions. Control samples were collected from healthy animals not vaccinated against brucellosis. Optimal preparation of bacterial antigen was achieved using sonication in lysis buffer so as to include a wide range of proteins including the membrane-bound ones. Samples of both species separated by SDS–PAGE exhibited a comparable number of signals, but differed in signal intensities due to unequal concentration of proteins. These differences might reflect bacterial specificity towards their host species.

Western blotting results indicated that all field sera contain antibodies reactive with at least 10 out of 18 bands distributed at 20, 30 and 70 kDa. Signals observed in the 70–100 kDa range appeared to be specific for the *B. abortus* proteins. No specific signals for proteins from *B. melitensis* were detected in this range. Using MALDI TOF-MS, proteins could be identified from 18 out of 22 bands analyzed (8 and 10 from *B. abortus* and *B. melitensis*, respectively); among the identified proteins we found heat shock proteins, enzymes, binding proteins and hypothetical proteins.
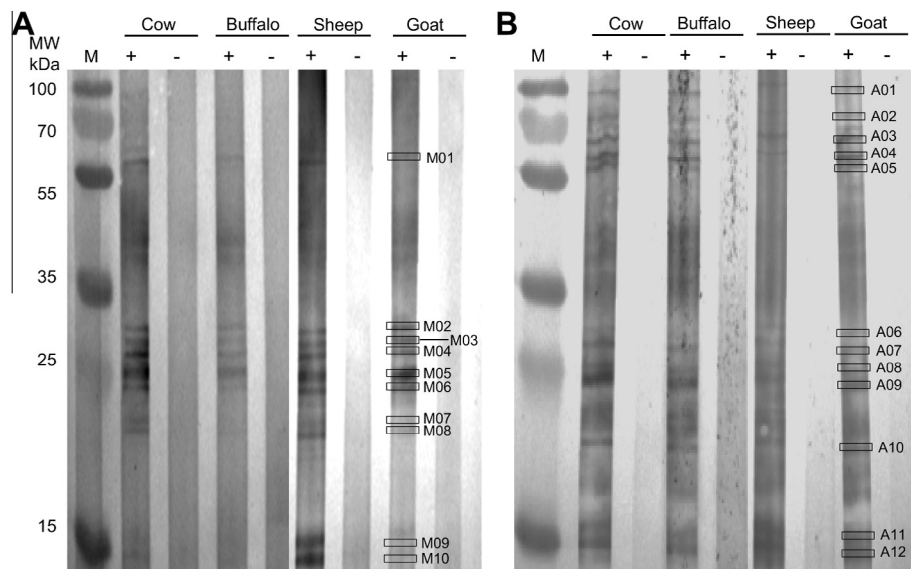
Fig. 2. Representative western blot images of *Brucella* whole-cell protein extracts separated on 12% polyacrylamide gel. The blot was developed using TMB kit after immunoblotting with sera from bovines and small ruminants and respective peroxidase-conjugated secondary antibodies. (A) Blot image of *B. melitensis*. (B) Blot image of *B. abortus*. M is the marker and the signs "+"and "−"represent the seropositive and seronegative serum samples, respectively.

Table 1
List of the proteins identified using immunoblotting and MALDI-TOF MS analysis.

Band ID	NCBI Acc. Nr.	Protein	MOWSE Score	MW (Da)	pI	Sera
A02	gi|493164348	Molecular chaperone DnaK	137	68,324	4.83	C,B,S,G
A03	gi|493174191	Molecular chaperone GroEL	98[a]	57,499	5.08	C,B,S,G
A04	gi|493053174	Catalase	236	57,519	6.62	C,B,S,G
A05	gi|493053174	Catalase	177	55,556	6.62	C,B,S,G
A06	gi|496220735	Ribose transport system substrate-binding protein	126	20,383	5.21	C,B,S,G
A08	gi|489054716	Transaldolase	156	23,631	5.07	C,B,S,G
A10	gi|493692656	Hypothetical protein	107	25,876	4.82	C,B,S,G
A12	gi|551701922	Superoxide dismutase, copper/zinc binding protein	131	16,176	6.11	C,B,S,G
M01	gi|493105393	Sn-glycerol-3-phosphate-binding periplasmic protein ugpB	77[a]	37,086	6.60	C,B,S,G
M02	gi|493690992	Amino acid ABC transporter substrate-binding protein	153	27,285	5.08	C,B,S,G
M03	gi|490822964	Glutamine amidotransferase	126	30,259	6.19	C,B,S,G
M04	gi|225851771	Fumarylacetoacetate hydrolase domain-containing protein 2	265	30,118	5.00	C,B,S,G
M05	gi|17988780	D-Ribose-binding periplasmic protein precursor	153	31,030	5.60	C,B,S,G
M06	gi|496765950	Hypothetical protein	162	24,587	4.72	C,B,S,G
M07	gi|493692656	Hypothetical protein	456	25,876	4.82	C,B,S,G
M08	gi|17986528	Translaldolase	228	23,620	5.69	C,B,S,G
M09	gi|384446825	Superoxide dismutase, copper/zinc binding protein	143	17,255	6.10	S
M10	gi|384446825	Superoxide dismutase, copper/zinc binding protein	290	17,255	6.10	S

Band ID: Prefix A refers to band from *B. abortus* and M refers to band from *B. melitensis*, NCBI Acc. Nr. is the accession number of the proteins identified by comparing the peptide sequence with NCBI databank, MOWSE score: The probability based MOWSE score is $-10 * Log(P)$, where P is the probability that the observed match is a random event. This list includes only those bands identified with MOWSE score greater than significant value ($p < 0.05$), MW: molecular weight calculated from identified protein sequence and pI: isoelectric point as calculated from the identified protein sequence, Sera: C = Cow, B = Buffalo, S = Sheep, G = Goat.

[a] MOWSE score is significant only with deprecated protein summary; hence, the data is not deposited at ProteomeXchange Consortium.

The sera from the naturally infected animals investigated in this study contain antibodies against all of the identified proteins indicating that the antigen exposure in all hosts is comparable or the same. It is not clear why only sheep sera possess antibodies against superoxide dismutase (SOD) from *B. melitensis*. In contrast all study sera contained antibodies against its homolog in *B. abortus*. Immunodominant proteins appear to be different between *B. abortus* and *B. melitensis*, for instance, heat shock proteins were detected only in *B. abortus*. The identified immunodominant proteins appear to be involved in bacterial survival during infection, however, it is not clear if these proteins contribute to host specificity.

Heat shock proteins (molecular chaperone DnaK and GroEL) have already been identified as antigenic proteins [12,13,16,24]. These proteins are mainly involved in protein formation (folding, assembly, and also transport of proteins) [25] and thereby correlate with the rapid turnover of proteins observed during the infection stage, bacterial survival and escape of the host immune system. DnaK is involved in rapid turnover of proteins for growth and for sustainability in the host cell environment [26]. In recent years, GroEL proved to be one of the *Brucella* immunodominant proteins [27]. In *B. abortus*, HSP60, a member of the GroEL family, aids the bacterial internalization process having an impact on the lipid rafts of the host cells [27].

SOD has been demonstrated to influence the oxidative environment of the host tissue, might inhibit host innate immune response and plays a role in intracellular survival [28]. Vaccination attempts using SOD resulted in partial protection only, but an immune response was still observed [29,30]. It has been demonstrated for *Mycobacterium tuberculosis* that SOD facilitates survival within macrophages and impairs immunologic function during early infection [31]. In case of catalase, the other antioxidant enzyme detected, it has already been shown to play no role in *Brucella* virulence [32]. Transaldolase is an important enzyme of the pentose phosphate pathway which was shown to have a protective effect against oxidative stress in yeast [33]. Secretion of transaldolase in *Bifidobacterium* was connected with the establishment and colonization in the intestinal tract [34]. Therefore, all these antioxidant enzymes might not have

an actual role in host specificity, but rather are beneficial for the survival of Brucellae in the host cell.

Two of the proteins identified were associated with cellular metabolism: Glutamine amidotransferase [35] is associated with biosynthesis of amino acids, nucleotides and coenzymes and fumarylacetoacetate hydrolase is associated with tyrosine degradation [36]. It has been proposed that adenine and guanine monophosphate can inhibit phagolysosome fusion [37]. Hence, glutamine amidotransferase might play a role in preventing phagolysosome fusion and for *Brucella* survival. For the time being, it remains unclear how these two intracellular proteins are processed by the host immune system for antibody generation.

The detection of immunoreactive binding proteins indicates the active transport of substances within the bacterium. Sn-glycerol-3-phosphate-binding periplasmic protein ugpB plays a direct role in the transport of biomolecules across the cell and D-ribose-binding periplasmic protein serves as the primary chemoreceptor for chemotaxis [38]. Though binding protein-dependent transport systems have been described in Gram-negative bacteria [39], the precise role of these binding proteins in *Brucella* infection or survival remains elusive.

As each single 1D SDS–PAGE band might represent more than one protein, further research should be carried out with western blotting after two-dimensional electrophoresis. There were some additional faint signals observed on the western blots that were not traceable on the Coomassie-stained gel, mainly due to sensitivity limitation of the method. These proteins could be identified using specially designed microarrays [8] with known proteins from both species. This might provide additional information on host specificity.

Furthermore, the production of antibodies in the host appears to be influenced to a high degree by the individual strain rather than the bacterial species itself [22]. The expression of immunodominant proteins is expected to be different between the *in vitro* and *in vivo* culture conditions. Therefore, the use of additional sera from various hosts representing different geographical regions and culturing the bacteria under conditions that mimic the host system might be helpful in understanding protein expression and the mechanism behind host specificity.

Acknowledgments

References

[1] J. Godfroid, H.C. Scholz, T. Barbier, C. Nicolas, P. Wattiau, D. Fretin, A.M. Whatmore, A. Cloeckaert, J.M. Blasco, I. Moriyon, C. Saegerman, J.B. Muma, S.A. Dahouk, H. Neubauer, J.-J. Letesson, Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century, Prev. Vet. Med. 102 (2011) 118–131.

[2] A. Whatmore, N. Davison, A. Cloeckaert, S. Al Dahouk, M. Zygmunt, S. Brew, L. Perrett, M. Koylass, G. Vergnaud, C. Quance, H. Scholz, E.J. Dick, G. Hubbard, N. Schlabritz-Loutsevitch, Brucella papionis sp. nov. isolated from baboons (Papio spp.), Int. J. Syst. Evol. Microbiol. (2014), http://dx.doi.org/10.1099/ijs.0.065482-0.

[3] E. Moreno, I. Moriyon, Brucella melitensis: a nasty bug with hidden credentials for virulence, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 1–3.

[4] M.N. Seleem, S.M. Boyle, N. Sriranganathan, Brucella: a pathogen without classic virulence genes, Vet. Microbiol. 129 (2008) 1–14.

[5] G. Gao, Y. Wang, Z. Chen, X. Xu, J. Xu, Brucella virulence mechanisms and implications in novel vaccines and drugs, Crit. Rev. Eukaryot. Gene Expr. 23 (2013) 49–64.

[6] J. Lamontagne, M. Béland, A. Forest, A. Côté-Martin, N. Nassif, F. Tomaki, I. Moriyón, E. Moreno, E. Paramithiotis, Proteomics-based confirmation of protein expression and correction of annotation errors in the Brucella abortus genome, BMC Genomics 11 (2010) 300.

[7] A.R. Wattam, K.P. Williams, E.E. Snyder, N.F. Almeida Jr., M. Shukla, A.W. Dickerman, O.R. Crasta, R. Kenyon, J. Lu, J.M. Shallom, H. Yoo, T.A. Ficht, R.M. Tsolis, C. Munk, R. Tapia, C.S. Han, J.C. Detter, D. Bruce, T.S. Brettin, B.W. Sobral, S.M. Boyle, J.C. Setubal, Analysis of ten Brucella genomes reveals evidence for horizontal gene transfer despite a preferred intracellular lifestyle, J. Bacteriol. 191 (2009) 3569–3579.

[8] L. Liang, D. Leng, C. Burk, R. Nakajima-Sasaki, M.A. Kayala, V.L. Atluri, J. Pablo, B. Unal, T.A. Ficht, E. Gotuzzo, M. Saito, W.J. Morrow, X. Liang, P. Baldi, R.H. Gilman, J.M. Vinetz, R.M. Tsolis, P.L. Felgner, Large scale immune profiling of infected humans and goats reveals differential recognition of Brucella melitensis antigens, PLoS Negl. Trop. Dis. 4 (2010) e673.

[9] S.M. Halling, B.D. Peterson-Burch, B.J. Bricker, R.L. Zuerner, Z. Qing, L.L. Li, V. Kapur, D.P. Alt, S.C. Olsen, Completion of the genome sequence of Brucella abortus and comparison to the highly similar genomes of Brucella melitensis and Brucella suis, J. Bacteriol. 187 (2005) 2715–2726.

[10] Y. Wang, Y. Ke, Z. Wang, X. Yuan, Y. Qiu, Q. Zhen, J. Xu, T. Li, D. Wang, L. Huang, Z. Chen, Genome sequences of three live attenuated vaccine strains of Brucella species and implications for pathogenesis and differential diagnosis, J. Bacteriol. 194 (2012) 6012–6013.

[11] M. Eschenbrenner, T.A. Horn, M.A. Wagner, C.V. Mujer, T.L. Miller-Scandle, V.G. DelVecchio, Comparative proteome analysis of laboratory grown Brucella abortus 2308 and Brucella melitensis 16M, J. Proteome Res. 5 (2006) 1731–1740.

[12] S. Al-Dahouk, K. Neockler, H.C. Scholz, H. Tomaso, R. Bogumil, H. Neubauer, Immunoproteomic characterization of Brucella abortus 1119-3 preparations used for the serodiagnosis of Brucella infections, J. Immunol. Methods 309 (2006) 34–47.

[13] J.P. Connolly, D. Comerci, T.G. Alefantis, A. Walz, M. Quan, R. Chafin, P. Grewal, C.V. Mujer, R.A. Ugalde, V.G. DelVecchio, Proteomic analysis of Brucella abortus cell envelope and identification of immunogenic candidate proteins for vaccine development, Proteomics 6 (2006) 3767–3780.

[14] Y.K. Kyung, J.-W. Kim, M. Her, S.-I. Kang, S.C. Jung, D.H. Cho, J.-Y. Kim, Immunogenic proteins of Brucella abortus to minimize cross reactions in brucellosis diagnosis, Vet. Microbiol. 156 (2012) 374–380.

[15] Y. Yang, L. Wang, J. Yin, X. Wang, S. Cheng, X. Lang, X. Wang, H. Qu, C. Sun, J. Wang, R. Zhang, Immunoproteomic analysis of Brucella melitensis and identification of a new immunogenic candidate protein for the development of brucellosis subunit vaccine, Mol. Immunol. 49 (2011) 175–184.

[16] Z. Zhao, F. Yan, W. Ji, D. Luo, X. Liu, L. Xing, Y. Duan, P. Yang, X. Shi, Z. Li, X. Wang, Identification of immunoreactive proteins of Brucella melitensis by immunoproteomics, Sci. China Life Sci. 54 (2011) 880–887.

[17] G.G. Alton, L.M. Jones, R.D. Angus, J.M. Verger, Techniques for the Brucellosis Laboratory, Institut national de la Recherche Agronomique, Paris, 1988. pp. 17–62.

[18] W.S. Probert, K.N. Schrader, N.Y. Khuong, S.L. Bystrom, M.H. Graves, Real-time multiplex PCR assay for detection of Brucella spp., B. abortus, and B. melitensis, J. Clin. Microbiol. 42 (2004) 1290–1293.

[19] J. Murugaiyan, M. Rockstroh, J. Wagner, S. Baumann, K. Schorsch, S. Trump, I. Lehmann, M.v. Bergen, J.M. Tomm, Benzo[a]pyrene affects Jurkat T cells in the activated state via the antioxidant response element dependent Nrf2 pathway leading to decreased IL-2 secretion and redirecting glutamine metabolism, Toxicol. Appl. Pharmacol. 269 (2013) 307–316.

[20] J. Murugaiyan, C. Weise, M.v. Bergen, U. Roesler, Two-dimensional proteome reference map of Prototheca zopfii revealed reduced metabolism and enhanced signal transduction as adaptation to an infectious life style, Proteomics 13 (2013) 2664–2669.

[21] J.A. Vizcaino, R.G. Cote, A. Csordas, J.A. Dianes, A. Fabregat, J.M. Foster, J. Griss, E. Alpi, M. Birim, J. Contell, G. O'Kelly, A. Schoenegger, D. Ovelleiro, Y. Perez-Riverol, F. Reisinger, D. Rios, R. Wang, H. Hermjakob, The PRoteomics IDEntifications (PRIDE) database and associated tools: status in 2013, Nucleic Acids Res. 41 (2013) D1063–D1069.

[22] Y. He, Analyses of Brucella pathogenesis, host immunity, and vaccine targets using systems biology and bioinformatics, Front. Cell Infect. Microbiol. 2 (2012), http://dx.doi.org/10.3389/fcimb.2012.00002 (eCollection 02012).

[23] A. Teixeira-Gomes, A. Cloeckaert, G. Bézard, G. Dubray, M.S. Zygmunt, Mapping and identification of Brucella melitensis proteins by two-dimensional electrophoresis and microsequencing, Electrophoresis 18 (1997) 156–162.

[24] D.R. Jones, S. Moussaud, P. McLean, Targeting heat shock proteins to modulate α-synuclein toxicity, Ther. Adv. Neurol. Disord. 7 (2014) 33–51.

[25] S. Köhler, E. Ekaza, J.Y. Paquet, K. Walravens, J. Teyssier, J. Godfroid, J.P. Liautard, Induction of dnaK through its native heat shock promoter is necessary for intramacrophagic replication of Brucella suis, Infect. Immun. 70 (2002) 1631–1634.

[26] A.Q. Abbady, A. Al-Daoude, A. Al-Mariri, M. Zarkawi, S. Muyldermans, Chaperonin GroEL a Brucella immunodominant antigen identified using Nanobody and MALDI-TOF-MS technologies, Vet. Immunol. Immunopathol. 146 (2012) 254–263.

[27] M.G. Stevens, S.C. Olsen, G.W. Pugh, J.E. Mayfield, Role of immune responses to a GroEL heat shock protein in preventing brucellosis in mice vaccinated with Brucella abortus strain RB51, Comp. Immunol. Microbiol. Infect. Dis. 20 (1997) 147–153.

[28] T.J. Break, S. Jun, M. Indramohan, K.D. Carr, A.N. Sieve, L. Dory, R.E. Berg, Extracellular superoxide dismutase inhibits innate immune responses and clearance of an intracellular bacterial infection, J. Immunol. 188 (2012) 3342–3350.

[29] L.B. Tabatabai, G.W.J. Pugh, Modulation of immune responses in Balb/c mice vaccinated with Brucella abortus Cu–Zn superoxide dismutase synthetic peptide vaccine, Vaccine 12 (1994) 919–924.

[30] D. Sáez, P. Fernández, A. Rivera, E. Andrews, A. Oñate, Oral immunization of mice with recombinant Lactococcus lactis expressing Cu, Zn superoxide dismutase of Brucella abortus triggers protective immunity, Vaccine 30 (2012) 1283–1290.

[31] D. Liao, Q. Fan, L. Bao, The role of superoxide dismutase in the survival of Mycobacterium tuberculosis in macrophages, Jpn. J. Infect. Dis. 66 (2013) 480–488.

[32] J.M. Gee, M.E. Kovach, V.K. Grippe, S. Hagius, J.V. Walker, P.H. Elzer, R.M.N. Roop, Role of catalase in the virulence of Brucella melitensis in pregnant goats, Vet. Microbiol. 102 (2004) 111–115.

[33] H. Juhnke, B. Krems, P. Kötter, K.D. Entian, Mutants that show increased sensitivity to hydrogen peroxide reveal an important role for the pentose phosphate pathway in protection of yeast against oxidative stress, Mol. Gen. Genet. 252 (1996) 456–464.

[34] I. González-Rodríguez, B. Sánchez, L. Ruiz, F. Turroni, M. Ventura, P. Ruas-Madiedo, M. Gueimonde, A. Margolles, Role of extracellular transaldolase from Bifidobacterium bifidum in mucin adhesion and aggregation, Appl. Environ. Microbiol. 78 (2012) 3992–3998.

[35] P. Mäntsälä, H. Zalkin, Glutamine amidotransferase function. Replacement of the active-site cysteine in glutamine phosphoribosylpyrophosphate amidotransferase by site-directed mutagenesis, J. Biol. Chem. 259 (1984) 14230.

[36] H. Pircher, G.D. Straganz, D. Ehehalt, G. Morrow, R.M. Tanguay, P. Jansen-Dürr, Identification of human fumarylacetoacetate hydrolase domain-containing protein 1 (FAHD1) as a novel mitochondrial acylpyruvase, J. Biol. Chem. 286 (2011) 36500–36508.

[37] M. Corbel, Brucellosis: an overview, Emerg. Infect. Dis. 3 (1997) 213–221.

[38] J.L. Garwin, J. Beckwith, Secretion and processing of ribose-binding protein in Escherichia coli, J. Bacteriol. 149 (1982) 789–792.

[39] C.F. Higgins, S.C. Hyde, M.M. Mimmack, U. Gileadi, D.R. Gill, M.P. Gallagher, Binding protein-dependent transport systems, J. Bioenerg. Biomembr. 22 (1990) 571–592.