Trends in Peptide and Protein Sciences
Volume 8 (2023): e7

DOI: https://doi.org/10.22037/tpps.v8i1.43825

Original Article

Immunomics Approach to Develop an Immunogenic Polypeptide from *Brucella abortus*: Design and Recombinant Expression

Mina Saadat[a], Masoud Gandomkar[a], Mojgan Bandehpour[a,b*], Mobina Bandehpour[b], Bahram Kazemi[b]

[a] *Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*

[b] *Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*

Article history:
Received: 7 November 2023
Accepted: 16 December 2023

Keywords:
Brucella abortus
Immunoproteomics
Recombinant
Protein

Mina Saadat:
https://orcid.org/0000-0002-9786-9807

Mojgan Bandehpour:
https://orcid.org/0000-0002-5479-2846

**Corresponding Author:*
Email: m.Bandehpour@sbmu.ac.ir
(M. Bandehpour)

HIGHLIGHTS

- Immunomics results of *Brucella abortus* demonstrated five immunogenic proteins.
- Antigenic epitopes were predicted and cloned in the pET22b expression vector.
- The expressed polypeptide was confirmed by interaction with antibodies in infected cattle's sera.

ABSTRACT

Brucellosis in livestock and its transmission to humans through the consumption of contaminated dairy products is an important issue. In this study, five immunogenic proteins were obtained from a 2DE gel analysis of *Brucella abortus* proteome after interaction with infected cattle's serum antibodies. These five proteins, MOXR family ATPase-α2, T9SS C-terminal Target domain-containing protein, Cobyric acid synthase, Hypothetical Protein, and Vir-B11 type IV Secretion Protein were introduced to the *Brucella abortus* proteome by MALDI-TOF MS/MS spectroscopy and then, analyzed with Mascot. Bioinformatics was applied to predict B and T cell epitopes, which were then randomly linked together to design a novel recombinant multi-epitope protein. The synthesized construct was transferred to *E. coli* BL21 and the expressed protein (ABOR) contained 549aa was confirmed with specific antibodies in infected cattle's serum.

Cite this article as: Saadat, M., Gandomkar, M., Bandehpour, M., Bandehpour, M. and B. Kazemi, (2023). Immunomics approach to develop an immunogenic polypeptide from *Brucella abortus*: Design and recombinant expression. *Trends Pept. Protein Sci.*, **8**: e7.

Introduction

Brucella species are facultative intracellular gram-negative bacteria categorized in the R-2 subgroup of the class proteobacteria, whose nomenclature is based on their pathogenicity and the reaction of the host immunity system (Verma et al., 2018; Suárez-Esquivel et al., 2020). Brucellosis, often called Malta fever, is a major worldwide zoonotic disease, causing infection in a broad spectrum of mammals, including humans, with an annual

incidence rate of about half a million people worldwide (Baldwin et al., 2006; Moosazadeh et al., 2016). Although cattle are the preferential host for *Brucella abortus* (*B. abortus*), it is one of the most infectious species of *Brucella* for humans (Skendros et al., 2011). Immunomics, the combination of proteomics and immunological approaches (such as genomics, transcriptomics, and bioinformatics), is currently favored over original antigen and vaccine finding methods that have proven insufficient for severe pathogens like the infective microbes of brucellosis (DE Sousa et al., 2016; Carvalho et al., 2016). The subsequent distinct selection and characterization of the seroreactivity to different proteins of *Brucella* could lead to an acceptable diagnostic test or the identification of sufficient recombinant protein for prophylactic approaches.

To begin the characterization, we first identify *B. abortus*-specific proteins reacting with circulating antibodies in their naturally preferred infected hosts (i.e., cattle) from an Iranian veterinary clinic. This pathogen generates different virulence factors that modify phagocytic susceptibility, cytokine production, cellular maturation, antigen presentation, and apoptosis.

The degree of protection proficiency depends on the potency of the candidate antigens to direct immunity toward a Th1-type response (Bahrami et al., 2020; Kaushik et al., 2010). In this study, the designed recombinant multi-epitope antigen named ABOR (*B. abortus* peptides with a linker) was expressed in *E. coli* BL21 (DE3) and purified. It was then evaluated in combination with chitin as an adjuvant with several immunity assays in guinea pigs to produce a model for veterinary vaccine investigations.

Materials and Methods

Preparation of the anti-sera and bacterial strain

To analyze the *B. abortus* bacterial strain, 12 cattle serum samples naturally infected with *Brucella* (containing specific antibodies), seven non-infected, and 7 cattle with *Mycobacterium* infection (the negative control) sera were given from the Meysam slaughterhouse (Tehran, Iran). Evaluation of serum samples was carried out using serological methods, including Rosebengal, Tube wright, and Coombs wright (Erfanian et al., 2013). The *B. abortus* strain was originally isolated and cultured from the axillary lymph nodes and placenta of naturally infected cattle that had recently been aborted. By separating the tissues in isolated and sterile conditions, crushing the tissues with a razor inside the laboratory plate was performed. Then, after washing with sterile 1X PBS buffer, the tissue pieces were cultured in *Brucella* broth culture medium (Merck, Germany) at 37 °C with 10% CO_2 for a maximum of 30 days. To confirm the genus and species of bacteria, one milliliter of the broth culture was centrifuged at 7800 g, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, USA).

The strains were identified by specific Polymerase Chain Reaction (PCR). The specific primers used were 16srDNAF: 5'ATATTGGACAATGGGCGCAA3' and 16srDNAR: 5'AGCGATTCCAACTTCATGCA3'. The amplified fragment was considered 958 bp of the *B. abortus* 16S rRNA gene. The PCR solution included 100 ng of DNA, 0.1mM dNTP, and 20 pmol each of forward and reverse primers, 1.5 mM $MgCL_2$, 1X PCR buffer, and 1.25 units of Taq DNA polymerase in a 30 µL final volume. The PCR program was considered under the following cycles: denaturing at 94 ºC for 30 s, annealing at 47 ºC for 30 s, and extension at 72 ºC for 45 s. These steps were repeated for 30 cycles. The reaction remained at 72 ºC for 5 min after PCR cycling.

B. abortus proteome extraction

To isolate the bacterial proteins, the identified strain was cultured in Tryptic Soy Broth (TSB) at 37 °C for 48 h while shaking (Hill et al., 2011; Watson et al., 2019) at the Razi Vaccine and Serum Research Institute (Karaj, Iran). Briefly, the culture was first centrifuged in the stationary growth phase, and after washing with phosphate buffer saline (PBS), the pellet was then incubated in lysis buffer (40 mM Tris base, 7 M urea, 2 M thiourea, and 0.001% bromophenol blue). Lastly, the samples were lysed by sonication on ice for 1 min (duty cycle: 0.5, amplitude: 80%), precipitated with acetone, and stored at -20 °C before use.

Bacterial protein isolation by two-dimensional polyacrylamide gel electrophoresis (2DE)

The isoelectric focusing (IEF) analysis was carried out using 7 cm immobilized pH gradient (IPG) strips with a nonlinear range of pH 3-10 (Bio-Rad). The IPG strips were rehydrated overnight by loading 0.5 mg of protein into a total volume of 200 µL of rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS, 0.2% Bio-Lyte pH 3-10, 50 mM Dithiothreitol (DTT)] and bromophenol blue. The focusing program for Protean IEF cell (Bio-Rad) regulated a linear increase from 0-250 V for 20 min, followed by 10,000 V, then fixing on it. Afterward, the IPG strips were equilibrated in a buffer (6 M urea, 50 mM Tris–HCl pH 8.8, 2% SDS, 25% glycerol, 0.01% color, and 2% DTT) for 20 min. Lastly, 2.5% iodoacetamide was used to alkylate the samples (20 min). In the second phase, equilibrated strips were placed on top of 12%

SDS-PAGE gel slabs. Electrophoresis was run using the standard Laemmli buffer system for approximately 6 h at 16 °C. Finally, a sensitive silver staining method was used to stain the gels.

Immune blotting with pooled sera from samples with brucellosis

For Western blotting, the 2D gel was blotted onto a nitrocellulose membrane (Sigma–Aldrich) using a semi-dry transfer cell (APELEX, U.K.). The membrane was suspended inside 3% skim milk at 25 ºC for 2 h. Pooled infected sera (1:200 diluted) were used as the primary antibody, while 1:10000 diluted Chicken anti-bovine IgG (Abcam, U.K.) peroxidase conjugate was used to detect *B. abortus* immunoreactive proteins. Spots were visualized using a substrate buffer (Diaminobanzoic acid), 10 mL of PBS containing 1% BSA, 0.05% Tween 20, and 30 µL of hydrogen peroxidase.

Protein identification by mass spectroscopy and MASCOT analysis

The protein sharp spots that reacted with the infected sera (in the pI 5 to 7 range) were excised from the gels and sent to the Proteomics International Company in Western Australia. Subsequently, a database exploration for protein characterization and verification was achieved using MS/MS ion search (MASCOT, matrixscience.com) against all entries of NCBInr (GenBank). The protein assessment was considered definitive if it matched more than two peptides for each protein, and the Molecular Weight Search **(**MOWSE) scores were significant ($P<0.05$).

Selection and retrieval of B. abortus proteins for chimeric immunogenic protein development

In the development of the immunogenic protein, it is crucial to integrate specific fragments of these factors. The proteins selected for this intention are shown in Table 1.

Prediction of B-cell epitopes performed using IEDB (http://tools.iedb.org) and ABCpred (http://crdd.osdd.net/raghava/abcpred/). To recognize T-cell epitopes several servers such as IEDB, ProPred (http://crdd.osdd.net/raghava/propred1/) with parameters for bovine MHC class II, DRA employed. The scanned B-cell and T-cell epitopes were connected using G.S. linkers to design a polypeptide, named ABOR.

Physical and chemical parameters including theoretical isoelectric point (pI), molecular weight, half-life, extinction coefficient, grand average of hydropathy (GRAVY) instability index, and the total number of residues, were computed using the ProtParam tool. (http://expasy.org/tools/protparam.html) .

Construction of pETabor22b plasmid (pET-22b vector cloned with abor)

After identifying the bacterial determinant proteins, the protein ABOR with 549aa was constructed with the epitopes from 5 selected proteins joined with G.S. linkers. The obtained protein was reverse-translated into DNA and amino acid codons were subsequently optimized based on *E. coli* BL21 codon usage by the JCAT server (http://www.JCAT.com/).

The efficiency of translation was assessed by structural stability and Gibbs free energy (ΔG) analysis. Finally, the *abor* gene was synthesized by the Generay company (Shanghai, China).

The ABOR recombinant protein was expressed by synthesizing the protein-coding sequence with restriction enzyme sites (1710 bp) into the pET22b (5493 bp) expression vector (GeneCust, Luxembourg S.A.) between the *SacI* and *HindIII* restriction enzyme sites. Restriction analysis confirmed the synthetic DNA sequence.

Expression, purification, and validation of ABOR protein

First, as the host cell, *E. coli* BL21 (DE3) was transformed with pETabor22b to produce the recombinant multi-epitope protein. The lysed bacterial pellet was electrophoresed onto a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) along with the protein marker (Fermentas, Lithuania). Then, the expression of the ABOR protein was compared with the control sample (*E. coli* BL21 without plasmid). Next, the recombinant protein was purified under native conditions with His-Tag resin (Invitrogen, Germany) according to the manufacturer's guidelines. Afterward, the purified ABOR polypeptide and the controls were lyophilized at a standard condition. The purified protein in 2µg/µL concentration was separated using the 12% SDS-PAGE and then transferred onto a nitrocellulose membrane (Sigma-Aldrich, U.K.) by blotting. The membrane was incubated for 90 min at 37 °C with 1:100 dilutions of cow's sera (obtained from 20 cows from the Razi Institute, previously confirmed by a serological test for brucellosis) to investigate the antigenicity of the purified protein. The goat anti-cow IgG horseradish peroxidase-conjugate (1:10000) (Abcam, U.K.) was put as the secondary antibody. Following the washing step, the protein bands were finally developed using DAB (diaminobenzidine) chromogen.

Results and Discussion

Following the introduction of various structural and secretory proteins of bacteria involved in its pathogenesis and the acquisition of antigens that stimulate the production of antibodies in the serum of infected cattle, these proteins can be confidently used to achieve the antigenic epitopes that we need to begin to build the proposed epitope-based vaccine. To assess bacterial proteins, we should confirm the isolated bacteria from infected animals as follows:

Isolation of B. abortus cells

The isolated bacteria were evaluated by specific PCR to cultivate and isolate the bacterial whole-cell proteins. Fig. 1 shows the amplification of the 16s rDNA fragment of *B. abortus* in tissue samples. According to previous studies, the chimeric recombinant proteins could create a multilateral impact and increase the protective effect of each specific antigen (Sieira et al., 2000; Gatkowska et al., 2019). Therefore, we believe that a multi-tope immunogenic protein containing immunodominant epitopes from several specific proteins may display sufficient immunity against *Brucella* species. In this regard, we employed Immunomics approaches to initiate the identification of suggestive immunogenic polypeptides for cattle brucellosis.

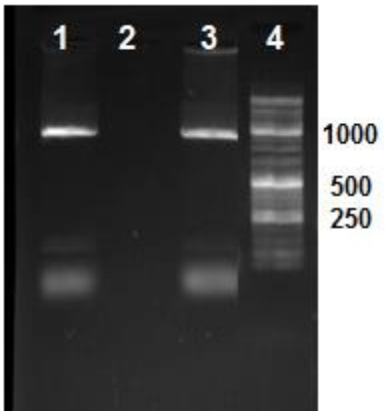

Figure 1. PCR product on 1% gel agarose electrophoresis. Lane 1: positive control. Lane 2: negative control. Lane 3: 958 bp PCR product of *B. abortus* 16s rDNA gene. Lane 4: DNA ladder marker.

Immunoreactive proteins of B. abortus cells

Western blotting of the bacterial strain of certified naturally infected sera showed that several antigenic proteins in *B. abortus* cells interact with the antibodies in the cattle serum (Fig. 2); this was not observed in the control samples.

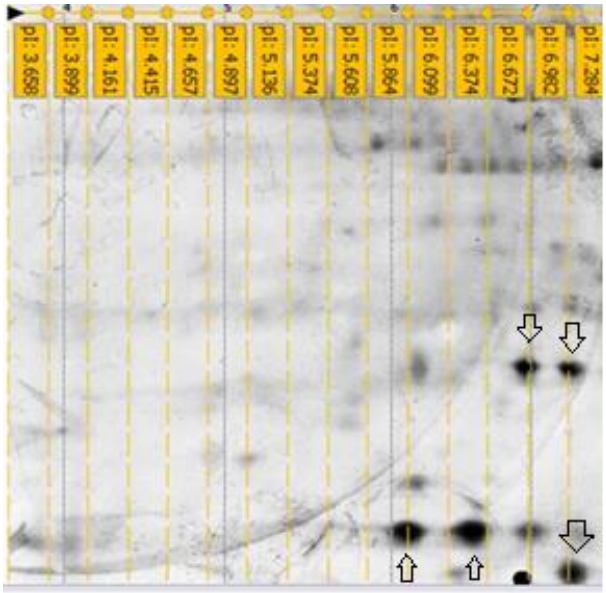

Figure 2. Western blot analysis of the 2D gel electrophoresis of cattle sera. The protein sharp spots that reacted with the infected sera (in the pI 5 to 7 range) in western blotting were excised from the gels. The interaction of proteins with pooled cattle sera was shown and analyzed with Melanie 9. The spots marked with the arrow in 5< pI <7 were considered for mass spectroscopy.

Proteomics analysis of B. abortus immunoreactive proteins

The protein spots that responded with infected sera were excised from the gels and prepared for analysis by MALDI-TOF MS/MS. MS/MS ion search (MASCOT, matrixscience.com) against all entries of NCBInr (GenBank) was used for a database exploration for protein recognition. The identified proteins (Table 1) were considered significant if two or more peptides were compliant and the MOWSE scores were valid ($P < 0.05$).

Construction of pETabor22b plasmid

We used a G.S. linker to join the immunogenic predicted epitopes to each other. The advantages of linkers are they provide enhanced biological actions, improved production, and plasma stability of tagged antibodies attaining a targeted or controlled drug transfer (Chen et al., 2013). Among all types of linkers, flexible types are generally implicated in small, non-polar, or polar residues such as Gly, Ser, and Thr. Flexible linkers are used for the advancement of protein folding with the epitope (Chakdar et al., 2016). The most common is the $(GlySer)_n$ linker. While polyG linkers have also been rated, the subtraction of a polar residue, such as serine, can save protein function.

Fig. 3 shows the pETabor-22b (7203 bp) expression vector including gene encoded the T-cell and B-cell

epitopes of *Brucella abortus* isolated proteins (*abor* gene) between HindIII and SacI cut sites. The figure illustrates the selected peptides based on each protein that is sorted in Table 1. Based on the DNA sequence frame in the vector, we added a codon for Methionine amino acid at the starting point of the reading frame. The ABOR poly-top protein was expressed in the prokaryotic *E.coli* system and purified by affinity chromatography.

Finally, because the cellular immune system is so important in the defense of the host body against *B. abortus*, five bacterial proteins were considered for epitope prediction and ABOR protein design. The designed poly-tope protein was called ABOR with 549aa, 47679.30 Da M.W., and pI 5.23. The protein included a total number of residues with negative (Asp+Glu) and positive (Arg+Lys) charges were 40 and 33, respectively. The estimated half-life of the protein is >10 h in *E. coli* and is considered a stable protein with an instability index of 38.77. The aliphatic index was 94.10, and the Grand average hydropathicity (GRAVY) was 0.159. Based on the comparative volume covered by aliphatic side chains (Ala, Val, ILe, and Leu), it may also be considered a thermostable protein.

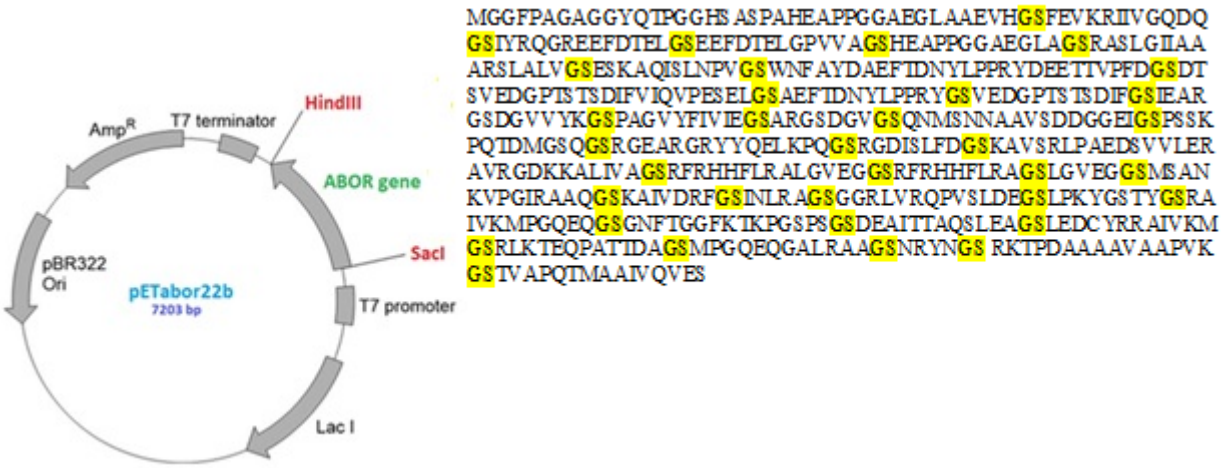

Figure 3. A schematic representation of the poly-epitope *abor* gene inserted into the pET-22b vector at the *SacI* and *HindIII* restriction sites. The *abor* multi-epitope sequence with G.S. linkers (Highlighted) connecting the predicted immunogenic epitopes.

Table 1: Identified proteins of *B. abortus* by the MALDI-TOF MS/MS and predicted epitopes.

Identified Proteins	pI	MW (kDa)	B cell epitopes	MHCI -T cell epitopes	MHCII -T cell epitopes
MOXR family ATPase-α2	6.98	110	GGFPAGAGGYQTPGGHSASPAHEAPPGGAEGLAAEVH FEVKRIIVGQDQ IYRQGREEFDTELG IEHEGVYPLPEAQRDR	EEFDTELGPVVA HEAPPGGAEGLA	RASLGIIAAARSLALV
T9SS C-terminal Target domain-containing protein	5.86	28	ESKAQISLNPV WNFAYDAEFTDNYLPPRYDEETTVPFD DTSVEDGPTSTSDIFVIQVPESEL	AEFTDNYLPPRY VEDGPTSTSDIF IEARGSDGVVYK	PAGVYFIVIEARGSDGV
Cobyric acid Synthase	6.40	30	QNMSNNAAVSDDGGEIG PSS KPQTDMGSQ RGEARGRYYQELKPQ RGDISLFD KAVSRLPAEDSVVLERAVRGDKKA LIVA	RFRHHFLRALGV EGG	RFRHHFLRALGVEGG
Hypothetical Protein	6.98	20	MSANKVPGIRAAQ	KAIVDRF	INLRA
VirB1 type IV Secretion Protein	7.25	100	GRLVRQPVSLDE LPKYGSTY RAIVKMPGQEQ GNFTGGFKTKPGSPS	DEAITTAQSLEA LEDCYRRAIVKM RLKTEQPATTDA MPGQEQGALRAA NRYN	RKTPDAAAAVAAPVK TVAPQTMAAIVQVES

Confirmation of the ABOR recombinant protein production

Western blot analysis of protein expression revealed by His-tag antibody and infected cattle's serum showed the target protein expression after induction, with a molecular weight of 47 kDa, which could interact with specific antibodies in the infected serum (Fig. 4).

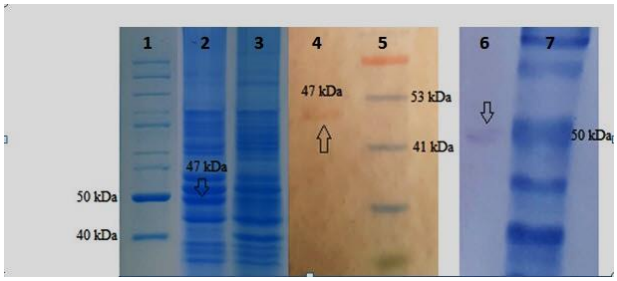

Figure 4. SDS-PAGE and western blotting of ABOR recombinant polytope. Lanes1, 5, and 7: MW Marker. Lane2: Induced *E. coli* lysate with 47 kDa protein extra band. Lane3: Purified ABOR protein by chromatography. Lane4: Demonstrated band of purified ABOR protein in interaction with specific antibodies in cattle serum in western blotting. Lane6: Expressed purified ABOR protein in interaction with His-Tag monoclonal antibody in western blotting.

Conclusion

In the present study, we have isolated five immunogenic proteins from *B. abortus* in interaction with infected animals' sera and identified them. In summary, the stable ABOR protein, a poly-tope substance with 549aa, developed from in silico prediction of antigenic epitopes. The aliphatic index and the Grand average hydropathicity (GRAVY) were 94.10 and 0.159, respectively. Finally, the antigenicity of the ABOR protein was confirmed by interaction with infected cattle's serum specific antibodies.

Acknowledgements

We would like to thank the staff at the Cellular and Molecular Research Center, Shahid Beheshti University of Medical Sciences.

Competing Interests

The authors have declared that they have no competing interests.

Ethical Statement

Following the ethical guidelines of the principles of research, this study was verified by the Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH. REC.1399.209). All of the raw data are available upon request.

Funding

This study was funded by Shahid Beheshti University of Medical Sciences, Cellular and Molecular Biology Research Center [grant number 43007351].

Authors Contribution

Mina Saadat and Mojgan Bandehpour designed and performed experiments, and co-wrote the paper. Mobina Bandehpour performed bioinformatics analyses. Bahram Kazemi supervised the research.

References

Bahrami, A.A., Bandehpour, M., Khalesi, B. and B. Kazemi, (2020). ″Computational design and analysis of a poly-epitope fusion protein: a new vaccine candidate for *Hepatitis* and *Poliovirus*.″ *International Journal of Peptide Research and Therapeutics*, **26**(1): 389-403. DOI: http://dx.doi.org/10.1007/s10989-019-09845-z.

Baldwin, C.L. and R. Goenka, (2006). ″Host immune responses to the intracellular bacteria *Brucella*: does the bacteria instruct the host to facilitate chronic infection?″ *Critical Reviews in Immunology*, **26**(5): 407-42. DOI: http://dx.doi.org/10.1615/CritRevImmunol.v26.i5.30.

Carvalho, T.F., Haddad, J.P., Paixão, T.A. and R.L. Santos, (2016). ″Meta-analysis and advancement of brucellosis vaccinology.″ *PloS One*, **11**(11): e0166582. DOI: http://dx.doi.org/10.1371/journal.pone.0166582.

Chen, X., Zaro, J.L. and W.C. Shen, (2013). ″Fusion protein linkers: property, design and functionality.″ *Advanced Drug Delivery Reviews*, **65**(10): 1357–69. DOI: http://dx.doi.org/10.1016/j.addr.2012.09.039.

Chakdar, H., Kumar, M., Pandiyan, K., Singh, A., Nanjappan, K., Kashyap, P.L. and A.K. Srivastava, (2016). ″Bacterial xylanases: biology to biotechnology.″ 3 *Biotech*, **6**(150): 1–15. DOI: http://dx.doi.org/10.1007/s13205-016-0457-z.

DE Sousa, K. and D. Doolan, (2016). ″Immunomics: a 21st century approach to vaccine development for complex pathogens.″ *Parasitol*ogy, **143**(2): 236-244. DOI: http://dx.doi.org/10.1017/S0031182015001079.

Erfanian, M., Seyyed Nouzadi, S. M. and L. Jarahi, (2013). ″Evaluation of diagnostic sensitivity of wright, coombs wright and 2-mercapto ethanol in diagnosis of Brucellosis.″ *Evidence Based Care*, **2**(4): 69-74. DOI: http://dx.doi.org/10.22038/ebcj.2013.488.

Gatkowska, J., Dzitko, K., Ferra, B.T., Holec-Gąsior, L., Kawka, M. and B. Dziadek, (2019). ″The impact of the antigenic composition of chimeric proteins on their immunoprotective activity against chronic toxoplasmosis in mice.″ *Vaccines*, **7**(4): 154. DOI: http://dx.doi.org/10.3390/vaccines7040154.

Hill, A.V. (2011). ″Vaccines against malaria.″ *Philosophical Transactions of the Royal Society B – Journals*, **366**(1579): 2806-14. DOI: http://dx.doi.org/10.1098/rstb.2011.0091.

Kaushik, P., Singh, D.K., Kumar, S.V., Tiwari, A.K., Shukla, G., Dayal, S. and P. Chaudhuri, (2010). ″Protection of mice against *Brucella abortus* 544 challenge by vaccination with recombinant OMP28 adjuvanted with CpG oligonucleotides.″ *Veterinary Research Communications*, **34**: 119-32. DOI: http://dx.doi.org/10.1007/s11259-009-9337-x.

Moosazadeh, M., Nikaeen, R., Abedi, G., Kheradmand, M. and S. Safiri, (2016). ″Epidemiological and clinical features of people with Malta fever in Iran: a systematic review and meta-analysis.″ *Osong Public Health and Research Perspectives*, **7**(3):157-67. DOI: http://dx.doi.org/10.1016/j.phrp.2016.04.009.

Sieira, R., Comerci, D.J., Sánchez, D.O. and R.A. Ugalde, (2000). ″A homologue of an operon required for DNA transfer in Agrobacterium is required in *Brucella abortus* for virulence and intracellular multiplication.″ *Journal of Bacteriology*, **182**(17): 4849-55. DOI: http://dx.doi.org/10.1128/JB.182.17.4849-4855.2000.

Skendros, P., Pappas, G. and P. Boura, (2011). ″Cell-mediated immunity in human brucellosis.″ *Microbes and Infection*, **13**(2): 134-42. DOI: http://dx.doi.org/10.1016/j.micinf.2010.10.015.

Suárez-Esquivel, M., Chaves-Olarte, E., Moreno, E. and C. Guzmán-Verri, (2020). ″*Brucella* genomics: macro and micro evolution.″ *International Journal of Molecular Sciences*, **21**(20): 7749. DOI: http://dx.doi.org/10.3390/ijms21207749.

Verma, S., Rawat, M., Kumawat, S., Qureshi, S., Mohd, G. and A.K. Tiwari, (2018). ″Protective role of *Brucella abortus*-specific murine antibodies in inhibiting systemic proliferation of virulent strain 544 in mice and guinea pig.″ *Veterinary World*, **11**(6): 794-9. DOI: http://dx.doi.org/10.14202/vetworld.2018.794-799.

Watson, P.S., Novy, P.L. and L.R. Friedland, (2019). ″Potential benefits of using a multicomponent vaccine for prevention of serogroup B meningococcal disease.″ *International Journal Infectious Diseases*, **85**: 22-7. DOI: http://dx.doi.org/10.1016/j.ijid.2019.05.019.

