January 2014

NOVEL STRATEGIES TO DEVELOP BETTER BRUCELLOSIS VACCINES USING BRUCELLA ABORTUS RB51 AND B. NEOTOMAE

Neha Dabral
Purdue University

Graduate School Form 30
(Revised 08/14)

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Neha Dabral

Entitled
NOVEL STRATEGIES TO DEVELOP BETTER BRUCELLOSIS VACCINES USING B. ABORTUS RB51 AND B. NEOTOMAE

For the degree of Doctor of Philosophy

Is approved by the final examining committee:

Dr. Ramesh Vemulapalli

Dr. Harm HogenEsch

Dr. Carolyn Guptill-Yoran

Dr. Mohamed Seleem

To the best of my knowledge and as understood by the student in the Thesis/Dissertation Agreement, Publication Delay, and Certification/Disclaimer (Graduate School Form 32), this thesis/dissertation adheres to the provisions of Purdue University's "Policy on Integrity in Research" and the use of copyrighted material.

Dr. Ramesh Vemulapalli

Approved by Major Professor(s): _____

_____

Approved by: Dr. Ramesh Vemupalli 11/14/2014

Head of the Department Graduate Program Date

NOVEL STRATEGIES TO DEVELOP BETTER BRUCELLOSIS VACCINES USING *BRUCELLA ABORTUS* RB51 AND *B. NEOTOMAE*

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Neha Dabral

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

December 2014

Purdue University

West Lafayette, Indiana

to

my parents, Piyush and Madhu Dabral

and my husband, Dinakar Sagapuram

ACKNOWLEDGEMENTS

I express my sincere gratitude to Dr. Ramesh Vemulapalli, my advisor, for his constant guidance, support and encouragement. He is an excellent mentor. He taught me the value of persistent hard work, self-motivation, and diligence. I thank him for his countless hours of encouraging and above all, patience. I deeply appreciate his dedication in improving my skills of scientific writing and critical analysis. Without his guidance, the completion of this thesis would never have been possible. His constructive criticism, profound insight into the subject, technical know-how and mentoring always guided me.

I thank my committee members Drs Harm HogenEsch, Carolyn Gutill-Yoran, and Mohamed Seleem, for their valuable time and suggestions on my research. I am sincerely grateful for their informative feedback and continuous support during my graduate school years. I am also thankful to Dr. Tracy Vemulapalli for her friendly discussions and encouragement.

I thank Dr Nammalwar Sriranganathan for his generous help with the protection studies and his helpful comments.

I am grateful to Dr. Dina Moustafa for her encouragement throughout my graduate studies. She deserves a special thanks for helping me get ‘unstuck’ with this thesis on many occasions. She knew when to push and when to let up. I cherish all the time spent with you and look forward to many more years of your companionship.

I thank my lab mate Marie Laoye for her friendship and constant company in and outside the workplace. Often, she provided a refreshing insight into the problems I encountered on this journey. I thank my friends Jamie, Amy, Yi, Fangjia, Shankar, Manish, Sriveny, Anurag, Yadvinder, Sai, Zhe, Seika, Heejoo, Jeong Ho, Haroon, Mohamed, Andrea, Naila, Kathy, Ana, Yung Yi, Grant, Keith. They made my graduate life an enjoyable and a

memorable experience. I also wish to thank Grant Burcham for his help with histology and Yi for her help with silver staining of the polyacrylamide gel.

I thank the Finks for their warm hospitality and for always making me feel welcome. They were my home away from home.

I am grateful to Barbara White for her help and cooperation. She always went above and beyond the call of duty for helping students and I was no exception. She was an invaluable asset to me to understand the workings of American academia. I will always and fondly remember the motherly affection she showered upon me. I also thank Carolyn and Matt for their generous help during my stay in the CPB department.

Special thanks to my best buddies, Reshu, Mert, Idil, Priyatham, Manny and Koko for being there through it all and making my life beautiful with their liveliness.

I thank my in laws, The Sagapurams, for their unconditional love, faith and support.

No words can express my gratitude to my parents for their love, support, encouragement and their blessings. Also, in myriad of ways in which, throughout my life, they have actively supported me in my determination to find and realise my potential. I thank my brother and sister-in-law, Paritosh and Vijayshree, for their affection and constant encouragement. I love you with all my heart.

Finally, I would like to thank my husband, Dinakar. In many deft ways, he lovingly challenged and supported me, and gave me courage throughout the whole of this work. Never a day goes by when I don’t appreciate having you in my life. I couldn’t have made it without you.

TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

ABSTRACT

Dabral, Neha, Ph.D., Purdue University, December 2014. Novel strategies to develop better brucellosis vaccines using *Brucella abortus* RB51 and *B. neotomae*. Major Professor: Ramesh Vemulapalli.

The genus *Brucella* consists of Gram-negative, facultative intracellular coccobacilli that can cause chronic infections in several mammals. *Brucella* spp. can exhibit a smooth or rough phenotype; smooth *Brucella* spp. contain a surface-exposed O-polysaccharide in their cell wall structure while the rough *Brucella* spp. are devoid of the O-polysaccharide. Acquired immunity against *Brucella* infection is primarily cell-mediated and involves both $CD4^+$ T cells and $CD8^+$ T cell responses. However, antibodies to the O-polysaccharide also play a role in enhancing the protection against infections by virulent *Brucella* species in some hosts. *B. abortus* strain RB51 is a stable rough attenuated mutant which is used as a licensed live vaccine for bovine brucellosis in the United States and several other countries. Previous studies have shown that the *wboA* gene, which encodes a glycosyltransferase required for the synthesis of O-polysaccharide in *Brucella*, is disrupted in *B. abortus* RB51 by an *IS711* element. Although low-levels of intra-cytoplasmic O-polysaccharide were produced when RB51 was complemented with a functional *wboA* gene (strain RB51WboA), it did not result in a smooth phenotype. This suggests that mutations in several genes of the O-polysaccharide biosynthesis pathway contribute to the rough phenotype of RB51. However, nucleotide sequence analysis has revealed that there are no other gene-disrupting mutations that could affect the smooth LPS synthesis in strain RB51. The first part of the study was undertaken to investigate whether overexpression of two other glycosyltransferases, WbkA and WbkE, in strain RB51 would result in the expression of O-polysaccharide and restore the smooth phenotype. No O-polysaccharide expression was detected on overexpression of *wbkA* or *wbkE* in RB51. However, *wbkA* overexpression

(strain RB51WbkA) leads to the development of extremely mucoid bacterial colonies that form clumps/strings in liquid culture. This mucoid phenotype is attributed to the production of exopolysaccharide(s) containing mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine. The clumping RB51WbkA strain exhibited increased adherence to polystyrene matrices; however, it was similar to strain RB51 in its attenuation characteristic and conferred a similar level of protection against virulent *B. abortus* 2308 as strain RB51. The second part of the study was carried out to determine whether increasing the amount of bactoprenol primed molecules in strain RB51WboA would lead to the expression of higher levels of O-polysaccharide and confer it a smooth phenotype. We generated strain RB51WboAKF by overexpressing *wbkF* gene, which encodes undecaprenyl-glycosyltransferase involved in bactoprenol priming for subsequent O-polysaccharide polymerization, in strain RB51WboA. Strain RB51WboAKF expressed high levels of O-polysaccharide and its Western blot reactivity profile with the O-polysaccharide-specific monoclonal antibody was similar to that of the smooth *Brucella* strains. Immunoelectron microscopy revealed that the O-polysaccharide was present mostly on the bacterial cell surface. However, RB51WboAKF strain exhibited rough phenotypic characteristic in acriflavine agglutination test. Although there was no difference between strains RB51 and RB51WboAKF in their ability to persist in spleens of BALB/c mice, strain RB51WboAKF was more resistant to the bactericidal effect of polymyxin B. Mice immunized with strain RB51WboAKF developed increased levels of smooth LPS-specific serum antibodies, primarily of IgG2a and IgG3 type, when compared with those immunized with strain RB51WboA. Levels of serum IL-12p70, IFN-γ and IL-10 were higher in mice immunized with strain RB51WboAKF when compared to the mice immunized with strain RB51. Splenocytes from the RB51WboAKF vaccinated group of mice secreted higher levels of antigen-specific IFN-γ, IL-10 and TNF-α when compared to those of the RB51 or RB51WboA vaccinated groups. Also, increased numbers of antigen-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T lymphocytes were detected in RB51WboAKF immunized mice. Importantly, immunization of mice with strain RB51WboAKF conferred greater protection against virulent *B. abortus* 2308 and *B. melitensis* 16M than immunization with RB51 or RB51WboA.

Human brucellosis is one of the most frequently encountered zoonotic diseases worldwide. It is a major public health concern in several developing, *Brucella*-endemic countries. Occupational exposure and consumption of infected dairy and meat products are the main routes for human infection. *B. abortus*, *B. melitensis*, and *B. suis* are the most frequent causes of disease in humans. These *Brucella* spp. are considered as potential weapons of biowarfare and are categorized by the CDC as Category B select agents. Emergence of new foci of zoonotic *Brucella* spp. around the world has significantly changed the global map of human brucellosis. Currently, there is no brucellosis vaccine available for use in humans. Safety concerns preclude the use of live attenuated animal vaccine strains for human vaccination.

The final part of the study was conducted to test the ability of orally inoculated gamma-irradiated strains *B. neotomae* and *B. abortus* RB51 in a prime-boost immunization approach to induce mucosal as well as systemic protection against virulent *B. abortus* 2308. Heterologous prime-boost vaccination regimens and homologous prime-boost vaccination of mice with *B. neotomae* led to the production of serum and mucosal antibodies specific to the smooth LPS. All oral vaccines induced antigen-specific $CD4^+$ and $CD8^+$ T cells capable of secreting IFN-γ and TNF-α. Upon intra-peritoneal challenge, mice vaccinated with *B. neotomae* showed the highest level of resistance against virulent *B. abortus* 2308 colonization in spleen and liver. Experiments with vaccination with different doses of *B. neotomae* showed that all tested doses of 10^9, 10^{10} and 10^{11} CFU-equivalent conferred significant protection against the intra-peritoneal challenge. However, only the highest tested dose of 10^{11} CFU-equivalent of *B. neotomae* afforded protection against intranasal challenge.

Overall, the present research discovered that gene *wbkA* is involved in exopolysachharide(s) production in *Brucella* spp. and adds to the growing evidence for the exopolysaccharide synthesis in *Brucella*. Also, our studies show that the vaccine efficacy of strain RB51 can be enhanced by inducing high levels of O-polysaccharide expression; the recombinant strain RB51WboAKF can be a more efficacious vaccine than its parent strain in natural hosts of *Brucella*. Finally, our study demonstrates the feasibility of using

gamma-irradiated *B. neotomae* as an effective and safe oral vaccine to induce mucosal and systemic protection against brucellosis.

INTRODUCTION

Bacteria belonging to the genus *Brucella* are Gram-negative, facultative intracellular, non-motile, non-encapsulated, non-spore forming aerobic coccobacilli that can cause brucellosis in several mammals, including humans. *Brucella* species are transmitted by direct contact with infected animal tissues and fluids or consumption of contaminated dairy and meat products. In natural animal hosts, *Brucella* spp. primarily target tissues of the reproductive tract and can cause abortion and infertility [1]. Till date, several species of *Brucella* have been identified and are classified based on, primarily, their host preference. Except for *B. canis* and *B. ovis*, all other *Brucella* species known to date possess an O-polysaccharide (O-PS) in their lipopolysaccharide (LPS) moiety and exhibit a smooth colony phenotype. Rough strains that are devoid of the O-PS in their LPS are generally attenuated than their smooth counterparts [2,3]. In the infected host, *Brucella* replicates within the phagosomes of macrophage-monocyte lineage cells. Despite the intricacy of microbicidal functions of professional phagocytes, *Brucella* possesses the ability to circumvent host defense mechanisms and ultimately acquire a replication-permissive niche by influencing the host intra-cellular vesicular pathway [4]. The ability of *Brucella* spp. to persist within host tissues is critical as natural transmission of *Brucella* only occurs once or twice annually during mating and parturition [5]. Interestingly, *Brucella* species lack classical bacterial virulence factors such as exotoxins, capsules, flagella, fimbriae, plasmids, lysogenic bacteriophages, antigenic variation and endotoxic LPS [6]. Instead, *Brucella* expresses a set of virulence factors, including a non-classical LPS molecule, which contribute to the establishment of chronic infections [4, 7]. Chapter 1 of this dissertation includes a review of the virulence determinants of *Brucella* and how they participate in its survival and trafficking within the host cells.

According to the World Health Organization (WHO), brucellosis is one of the most frequently encountered zoonosis worldwide [8]. Countries of the Mediterranean basin, Middle East, Central Asia, South America and Africa have a high prevalence of brucellosis [8]. Occupational exposure and consumption of contaminated food products are the main routes of human infection. Most of the naturally occurring human infections are caused by *B. melitensis, B. abortus* and *B. suis*. A few human brucellosis cases are caused by *B. canis* that is associated with brucellosis in dogs [9]. Due to their highly infectious nature and ease of aerosol spread, *B. melitensis, B. suis* and *B. abortus* are considered as potential bioweapons and classified as Class B select agents by the Center for Disease Control (CDC) [9]. Infected humans exhibit flu-like symptoms including general malaise, anorexia, headache, myalgia, arthralgia, fever. If left untreated, the infection can lead to a chronic disease and in rare cases complications like endocarditis, meningitis and spondylitis can occur [9]. Successful treatment of human disease relies on a prolonged therapy with a combination of antibiotics (usually an aminoglycoside or rifampicin and tetracycline); however, relapses are common [10,11]. There is no brucellosis vaccine available for use in humans.

Humans are dead-end hosts of *Brucella*; a reduction in animal brucellosis prevalence will result in a substantially reduced number of human infections. Through concerted comprehensive efforts, several countries including the United States, Canada and parts of Europe have succeeded in controlling brucellosis in domestic livestock and are considered to be 'brucellosis-free' [8]. However, there is a constant threat of *Brucella* re-introduction due to spillback from wild life reservoirs. Also, worldwide emergence of new foci of human brucellosis has made *Brucella* eradication difficult [12]. Human brucellosis remains a major public health concern in several developing countries that still suffer with a huge disease burden in animals and humans [13]. Due to differences in livestock production systems as well as economic and social constraints, control measures that are effective in developed countries may not be implementable in developing countries. For this reason, vaccination is considered to be the most cost-effective intervention to control disease spread and limit its severity [14]. Currently, live attenuated vaccines are used to prevent brucellosis in livestock. Smooth strains *B. melitensis* Rev 1 and *B. abortus* S19 are

used for vaccinating sheep and goats, and cattle, respectively [15,16]. However, these strains exhibit some abortifacient potential in pregnant animals and are virulent for humans [17]. A rough strain *B. abortus* RB51 is used as a licensed vaccine for cattle in the United States and several other countries. It is a stable, attenuated, spontaneous mutant of the virulent strain *B. abortus* 2308 [18]. Nevertheless, this vaccine is only 70% efficacious in cattle and its efficacy in pigs, sheep and goats is questionable [19-21]. Safety concerns preclude the use of these live attenuated strains for human use. A human vaccine would be valuable as a means of direct intervention in areas where natural infections are very high or in the event of deliberate misuse of *Brucella* as a bioweapon. Previously, numerous attempts were made to vaccinate humans with live attenuated strains and 'non-living' subcellular fractions in the former USSR, China and France [22-24]. However, the use of these vaccines was discontinued on account of safety issues and lack of substantial evidence of their efficacy from large, well-controlled clinical trials [25]. A concise history of human vaccination and strategies that need to be incorporated in any future efforts to develop a safe and effective vaccine against human brucellosis are discussed in Chapter 2. Despite the availability of live attenuated vaccines for ruminants, there is a need for developing a new generation of improved vaccines. A safe vaccine that can provide long-lasting protection in multiple animal species would be ideal for controlling brucellosis. Several previous studies have demonstrated that exposure to an optimum dose of gamma-rays can enhance the safety of the current live *Brucella* vaccine strains by abolishing the replicative ability of the bacteria [26-28]. Minimum exposure to gamma-rays creates sufficient number of breaks in the genome that makes the bacteria unable to replicate but still contain a large portion of the genomic nucleic acid intact for transcriptional and translational activity [26-28]. This ensures the downstream effectiveness of elicited host immune responses against the expressed proteins of the pathogen. Gamma-irradiated vaccines are metabolically active [29]; metabolic activity is a potential pathogen-associated molecular pattern (PAMP) that is associated with microbial viability (vita-PAMP) and is exploited by the host defense system to differentiate viable pathogens from killed pathogens [30]. vita-PAMP signal allows the host defense system to scale the level of microbial threat and deploy a more robust immune response against viable organisms [31].

Hence, while possessing the safety profile of killed vaccines, gamma-irradiated vaccines are capable of inducing protection at levels comparable to live vaccination strategies [26-28]. A review of the role of gamma-rays in the development of replication-deficient safer vaccines that stimulate effective protection is presented in Chapter 3.

Cell-mediated immune responses play a major role in acquired immunity against *Brucella*. In particular, IFN-γ-secreting $CD4^+$ and $CD8^+$ T lymphocytes are crucial for controlling *Brucella* infection [32,33]. At least in certain animal species, O-PS-specific antibodies also participate in protection against *B. melitensis*, *B. abortus* and *B. suis* infection [34-37]. In contrast to smooth vaccines Rev 1 and S19, the rough vaccine strain RB51 does not induce detectable levels of anti-O-PS antibodies in the vaccinated hosts [18]. Previous studies have demonstrated that *wboA* gene, which encodes a glycosyltransferase required for O-PS synthesis in *Brucella*, is disrupted by an *IS711* element in strain RB51 [38]. Absence of O-PS-specific antibodies prevent the development of cross-reacting immune responses in the serodiagnostic assays that are used for distinguishing infected animals from vaccinated animals (DIVA strategy). However, the absence of O-PS and the rough LPS make strain RB51 more sensitive to complement-mediated lysis, limiting its persistence in the host and vaccine efficacy [39,40]. Complementation of RB51 with a functional *wboA* gene (strain RB51WboA) did not restore the smooth phenotype but resulted in low levels of cytoplasmic O-PS expression [41]. When compared with strain RB51, immunization of mice with RB51WboA resulted in a superior level of protection against virulent *B. abortus* 2308 and *B. melitensis* 16M [41,42]. Several genes in the *wbk* and *wbo* genetic loci are shown to be essential for the O-PS synthesis in *Brucella* [2,3]. The objectives of the studies in Chapters 4 and 5 were to enhance the vaccine efficacy of strain RB51 through recombinant engineering to induce high levels of O-PS expression. To achieve this, we overexpressed selected genes of the O-PS biosynthetic pathway in strain RB51 by transforming RB51 with a multi copy plasmid containing the specific *Brucella* gene under a strong synthetic promoter. We hypothesized that such recombinant vaccines would possess enhanced protective efficacy when compared with strain RB51 due to their ability to stimulate O-PS-specific antibodies in the vaccinated animals. Studies to determine whether overexpression of two glycosyltransferases, WbkA and WbkE, in strain RB51

would result in the synthesis of O-PS and confer it a smooth phenotype are described in Chapter 4. The studies in Chapter 5 were carried out to determine whether increasing the amount of bactoprenol priming precursor molecules in strain RB51WboA would increase the level of O-PS expression and result in smooth phenotype of the recombinant bacteria. For this purpose, we overexpressed *wbkF*, which is involved in priming bactoprenol for O-PS polymerization [2,3], in strain RB51WboA. Also, the immunogenicity and the protective efficacy of the constructed strain was evaluated in the mouse model of brucellosis.

The studies in Chapter 6 were aimed at developing an oral vaccine against brucellosis for use in humans. *Brucella* infection is generally acquired through mucosal routes; however, human brucellosis is a systemic disease. Accordingly, it is important that an effective oral vaccine should induce both mucosal and systemic immunity. Oral vaccines have proved effective in providing protection against diseases like typhoid [43] and polio [44]. An attenuated *Salmonella enterica* serovar Typhi strain, which is used as a licensed oral vaccine against typhoid fever in humans, requires 4 doses to be taken at prescribed alternate day intervals [45]. Using a similar empirical selection, experiments in Chapter 6 were conducted to identify the optimum prime-boost vaccine-dose approach that would afford the highest level of protection against parenteral as well as mucosal challenge with virulent *Brucella*. Most research pertaining to prime-boost approach has been directed towards the assessment of DNA vaccines, viruses or purified proteins as immunogens. However, it is well accepted that vaccines that mimic the antigenicity of live infectious organisms have greater likelihood to possess better protective efficacy as they would be able to stimulate all facets of the immune system. Therefore, we used gamma-irradiated strains *B. abortus* RB51 and *B. neotomae* in various prime-boost combinations and assessed the induction of mucosal and systemic immunity. Previous studies have demonstrated that intra-peritoneal (i.p) vaccination with gamma-irradiated RB51 or gamma-irradiated *B. neotomae* protects mice against i.p challenge with virulent *Brucella* species [28,29]. In general, parenteral vaccination strategies do not afford protection at mucosal surfaces [46]. We hypothesized that oral delivery of gamma-irradiated *Brucella* strains would prime the host immune system for subsequent mucosal as well as systemic recall responses leading to enhanced

protection at both sites. Inducing a robust mucosal immunity would enhance resistance to brucellosis by limiting bacterial invasion through the primary route of infection.

The final Chapter 7 summarizes the conclusions of the present research and discusses their implications. Also, potential areas of future work are discussed. Overall, the present research provides evidence that vaccination with recombinant *Brucella abortus* RB51 strain engineered to express increased levels of O-PS provides enhanced protection against virulent *Brucella* challenge. Also, it demonstrates the feasibility of using gamma-irradiated strain *B. neotomae* as an oral vaccine against parenteral as well as mucosal *Brucella* infection.

References

[1] Carvalho Neta, A. V., Mol, J. P., Xavier, M. N., Paixao, T. A., Lage, A. P., Santos, R. L., 2010. Pathogenesis of bovine brucellosis. Vet J 184, 146-155.

[2] Gonzalez, D., Grillo, M. J., de Miguel, M. J., Ali, T., Arce-Gorvel, V., Delrue, R. M., Conde-Alvarez, R., Munoz, P., Lopez-Goni, I., Iriarte, M., Marin, C. M., Weintraub, A., Widmalm, G., Zygmunt, M., Letesson, J. J., Gorvel, J. P., Blasco, J. M., Moriyon, I., 2008. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLoS One 3, e2760.

[3] Zygmunt, M. S., Blasco, J. M., Letesson, J. J., Cloeckaert, A., Moriyon, I., 2009. DNA polymorphism analysis of *Brucella* lipopolysaccharide genes reveals marked differences in O-polysaccharide biosynthetic genes between smooth and rough *Brucella* species and novel species-specific markers. BMC Microbiol 9, 92-105.

[4] Pizarro-Cerda, J., Moreno, E., Sanguedolce, V., Mege, J. L., Gorvel, J. P., 1998. Virulent *Brucella abortus* avoids lysosome fusion and distributes within autophagosome-like compartments. Infect Immun 66, 2387-2392.

[5] De Jong, M. F., Tsolis, R. M., 2012. Brucellosis and type IV secretion system. Fut Microbiol 7, 47-58.

[6] Moreno, E., Moriyo´n, I., 2001. The genus *Brucella*. In: The Prokaryotes. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K. H., Stackebrandt, E. (Eds.), Electronic Version, Springer, New York.

[7] Lapaque, N., Moriyon, I., Moreno, E., Gorvel, J. P., 2005. *Brucella* lipopolysaccharide acts as a virulence factor. Curr Opin Microbiol 8, 60-66.

[8] Pappas, G., Panagopoulou, P., Akritidis, N., Christou, L., Tsianos, E. V., 2006b. The new global map of human brucellosis. Lancet Infect Dis 6, 91-99.

[9] Young, E. J., 2000. *Brucella* species. In: Principles and Practice of Infectious Disease, 5th ed., Mandel, G. L., Bennett, J. E., Dolin, R. (Eds.), Churchill-Livingstone, Philadelphia, PA, 2386–2393.

[10] Memish, Z., Mah, M. W., Al Mahmoud, S., Al Shaalan, M., Khan, M. Y., 2000. *Brucella* bacteraemia: clinical and laboratory observations in 160 patients. J Infect 40, 59-63.

[11] Hall, W. H., 1990. Modern chemotherapy for brucellosis in humans. Rev Infect Dis 12, 1060-99.

[12] Whatmore, A. M., Dawson, C. E., Groussaud, P., Koylass, M. S., King, A. C., Shankster, S. J., 2008. Marine mammal *Brucella* genotype associated with zoonotic infection. Emerg Infect Dis 14, 517–518.

[13] Cutler, S. J., Whatmore, A. M., Commander, N. J., 2005. Brucellosis- new aspects of an old disease. J Appl Microbiol 98, 1270-1281.

[14] WHO, 2005. Immunization against diseases of public health importance. In Fact Sheets: No. 288.

[15] Nicoletti, P., 1990. Vaccination. In: Nielsen, K., Duncan, J. R. (Eds.), Animal Brucellosis. CRC Press, Boca Raton, 284-299.

[16] Elberg, S. S., Faunce, W. K., 1957. Immunization against *Brucella* infection. IV. Immunity conferred on goats by a non-dependent mutant from a streptomycin dependent mutant strain of *Brucella melitensis*. J Bacteriol 73, 211-217.

[17] Spink, W. W., Hall, J. W. 3rd., Finstad, J., Mallet, E., 1962. Immunization with viable *Brucella* organisms. Results of a safety test in humans. Bull World Health Organ 26, 409-419.

[18] Schurig, G. G., Roop, R. M., Bagchi, T., Boyle, S., Buhrman, D., Sriranganathan, N., 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 28, 171-188.

[19] Jimenez de Bagues, M. P., Barberan, M., Marin, C. M., Blasco, J. M., 1995. The *Brucella abortus* RB51 vaccine does not confer protection against *Brucella ovis* in rams. Vaccine 13, 301-304.

[20] Olsen, S. C., Cheville, N. F., Stevens, M. G., Houng, H. H., Drazek, E. S., Hadfield, T. L., Warren, R. L., Hoover, D. L., 1997. Lymphocyte proliferative responses of goats vaccinated with *Brucella melitensis* 16M or a delta purE201 strain. Infect Immun 65, 2987-2991.

[21] Roop, R. M. 2nd., Jeffers, G., Bagchi, T., Walker, J., Enright, F. M., Schurig, G. G., 1991. Experimental infection of goat fetuses in utero with a stable, rough mutant of *Brucella abortus*. Res Vet Sci 51, 123-127.

[22] Kolar, J., 1977. *Brucella* vaccines production in Mongolia. Assignment report on WHO project MOG BLG 001, SEA/Vaccine/89. WHO, 40.

[23] Vershilova, P. A., 1961. The use of live vaccine for vaccination of human beings against brucellosis in the USSR. B World Health Organ 24, 85–89.

[24] Lopez-Merino, A., Asselineau, J., Serre, A., Roux, J., Bascoul, S., Lacave, C., 1976. Immunization by an insoluble fraction extracted from *Brucella melitensis*: immunological and chemical characterization of the active substances. Infect Immun 13, 311-321.

[25] Hadjichristodoulou, C., Voulgaris, P., Toulieres, L., Babalis, T., Manetas, G., Goutziana, G., Kastritis, I., Tselentis, I., 1994. Tolerance of the human brucellosis vaccine and the intradermal reaction test for brucellosis. Eur J Clin Microbiol 13, 129–134.

[26] Datta, S. K., Okamoto, S., Hayashi, T., Shin, S. S., Mihajlov, I., Fermin, A., Guiney, D. G., Fierer, J., Raz, E., 2006. Vaccination with irradiated *Listeria* induces protective T cell immunity. Immunity 25, 143-152.

[27] Magnani, D. M., Harms, J. S., Durward, M. A., Splitter, G. A., 2009. Nondividing but metabolically active gamma-irradiated *Brucella melitensis* is protective against virulent *B. melitensis* challenge in mice. Infect Immun 77, 5181-5189.

[28] Sanakkayala, N., Sokolovska, A., Gulani, J., Hogenesch, H., Sriranganathan, N., Boyle, S. M., Schurig, G. G., Vemulapalli, R., 2005. Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant *Brucella abortus* RB51. Clin Diag Lab Immunol 12, 1429-1436.

[29] Moustafa, D., Garg, V. K., Jain, N., Sriranganathan, N., Vemulapalli, R., 2011. Immunization of mice with gamma-irradiated *Brucella neotomae* and its recombinant strains induces protection against virulent *B. abortus, B. melitensis* and *B. suis* challenge. Vaccine 29, 784-794.

[30] Blander, J. M., Sander, L. E., 2012. Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. Nat Rev Immunol 12, 215-225.

[31] Sander, L. E., Davis, M. J., Boekschoten, M. V., Amsen, D., Dascher, C. C., Ryffel, B., Swanson, J. A., Muller, M., Blander, J. M., 2011. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. Nature 474, 385-389.

[32] Murphy, E. A., Sathiyaseelan, J., Parent, M. A., Zou, B., Baldwin, C. L., 2001. Interferon-gamma is crucial for surviving a *Brucella* infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunology 103, 511-518.

[33] Yingst, S., Hoover, D. L., 2003. T cell mediated immunity to brucellosis. Crit Rev Microbiol 29, 313-331.

[34] Araya, L. N., Winter, A. J., 1990. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infect Immun 58, 254-256.

[35] Ficht, T. A., Kahl-Mcdonagh, M. M., Arenas-Gamboa, A. M., Rice-Ficht, A. C., 2009. Brucellosis: the case for live, attenuated vaccines. Vaccine 27, D40-D3.

[36] Montaraz, J. A., Winter, A. J., Hunter, D. M., Sowa, B. A., Wu, A. M., Adams, L. G., 1986. Protection against *Brucella abortus* in mice with O-polysaccharide-specific monoclonal antibodies. Infect Immun 51, 961-963.

[37] Vitry, M. A., Mambres, D. H., Trez, C. D., Akira, S., Ryffel, B., Letesson, J. J., Muraille, E., 2014. Humoral immunity and $CD4^+$ Th1 cells are both necessary for a fully protective immune response upon secondary infection with *Brucella melitensis*. J Immunol 192, 3740-3752.

[38] Vemulapalli, R., McQuiston, J. R., Schurig, G., Sriranganathan, N., Halling, S. M., Boyle, S. M., 1996. Identification of an *IS711* element interrupting the *wboA* gene of *Brucella abortus* strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. Clin Diagn Lab Immunol 6, 760-764.

[39] Eisenschenk, F. C., Houle, J. J., Hoffmann, E. M., 1995. Serum sensitivity of field isolates and laboratory strains of *Brucella abortus*. Am J Vet Res 56, 1592-1598.

[40] Allen, C. A., Adams, L. G., Ficht, T. A., 1998. Transposon-derived *Brucella abortus* rough mutants are attenuated and exhibit reduced intracellular survival. Infect Immun 66, 1008-1016.

[41] Vemulapalli, R., He, Y., Buccolo, L. S., Boyle, S. M., Sriranganathan, N., Schurig, G., 2000. Complementation of *Brucella abortus* RB51 with a functional *wboA* gene results in O-antigen synthesis and enhanced vaccine efficacy but no change in rough phenotype and attenuation. Infect Immun 68, 3927-3932.

[42] Vemulapalli, R., Contreras, A., Sanakkayala, N., Sriranganathan, N., Boyle, S. M., Schurig, G. G., 2004. Enhanced efficacy of recombinant *Brucella abortus* RB51 vaccines against *B. melitensis* infection in mice. Vet Microbiol 102, 237-245.

[43] Guzman, C. A., Borsutzky, S., Griot-Wenk, M., Metcalfe, I. C., Pearman, J., Collioud, A., Favre, D., Dietrich, G., 2006. Vaccines against typhoid fever. Vaccine 24, 3804-3811.

[44] Kew, O. M., Sutter, R. W., Gourville, E. M., Dowdle, W. R., Pallansch, M. A., 2005. Vaccine-derived polioviruses and the endgame strategy for global polio eradication. Annu Rev Microbiol 59, 587-635.

[45] Dietrich, G., Griot-Wenk, M., Metcalfe, I. C., Lang, A. B., Viret, J. F., 2003. Experience with registered mucosal vaccines. Vaccine 21, 678-683.

[46] Neutra, M. R., Kozlowski, P. A., 2006. Mucosal vaccines: the promise and the challenge. Nat Rev Immun 6, 148-158.

CHAPTER 1. *BRUCELLA* AND ITS VIRULENCE DETERMINANTS

Abstract

Members of the bacterial genus *Brucella* are facultative intracellular pathogens that reside predominantly within macrophages of infected hosts. The capacity of *Brucella* to persist within the phagosomes of macrophages is critical to their ability to produce chronic infections in their mammalian hosts. Within macrophages, *Brucella* species regulate their intracellular trafficking to avoid degradation along the endocytic pathway. A precise sequence of programmed intracellular events allow *Brucella* to efficiently subvert host defense mechanisms and create a niche that permits replication while protecting them from microbicidal functions of host cells. This sophisticated lifestyle of *Brucella* is strongly dependent on the expression of several bacterial factors that are crucial to its protracted survival within the host system. In this chapter, we review the virulence determinants of *Brucella* and how they contribute to its pathogenicity.

Introduction

Brucella genus belongs to the α-2 subclass of the *Proteobacteria* that also includes several other phylogenetically related plant pathogens, symbionts and intracellular animal pathogens such as *Bartonella*. *Brucella* spp. are Gram-negative, aerobic, non-motile, nonsporulating, facultative intracellular coccobacilli that primarily replicate in the monocyte-macrophage lineage of host cells. They are the causative agents of brucellosis, a chronic disease of humans and several animal species. In pregnant animals, *Brucella* also

replicates in placental trophoblasts leading to frequent contagious abortions [1]. Transmission of *Brucella* occurs through direct contact with contaminated aborted fetal tissues or consumption of contaminated meat and dairy products. Human brucellosis is strictly a zoonotic disease; the incidence of human disease is dependent on the prevalence of animal brucellosis.

The genus *Brucella* is classified into several species based on their host preference. Natural hosts for *Brucella* species are: cattle (*B. abortus*), sheep and goats (*B. melitensis*), swine (*B. suis*), dogs (*B. canis*), rams (*B. ovis*), desert wood rats (*B. neotomae*), seals (*B. ceti*), dolphins, porpoises and whales (*B. pinnipedialis*) and common voles (*B. microti*) [2]. More recently, *Brucella* has been isolated from baboons (*B. papionis*), Australian rodents, and an infected breast implant (*B. inopinata*) [3,4,5]. Due to their highly infectious nature and the ease of aerosol spread, *B. melitensis, B. suis,* and *B. abortus* are considered as potential biowarfare agents and are classified as Class B pathogens by the Centers of Disease Control (CDC).

Brucella lacks the well-known classical bacterial virulence factors such as capsules, exotoxins, lysogenic bacteriophages, plasmids, flagella, fimbriae, antigenic variation and endotoxic LPS [6]. The virulence of *Brucella* relies on its ability to modulate its trafficking within host cells to avoid the formation of a degradative phagolysosome. Bacterial factors, such as lipopolysaccharide (LPS) [7], type IV secretion system (T4SS) [8], and the BvrR/BvrS two-component system [9] are essential for the intracellular lifestyle of *Brucella* and therefore, they are considered as virulence factors of these pathogens. Although these virulence determinants may not directly mediate clinical symptoms of brucellosis, they have been identified to be critical for *Brucella* to survive and replicate in the vacuolar phagocytic compartment of macrophages. This chapter reviews the molecular mechanisms underlying intracellular trafficking of *Brucella* and the virulence factors that contribute to *Brucella* persistence.

Invasion and intracellular trafficking of *Brucella* in phagocytic cells

Brucella are mostly transmitted through mucosal routes, especially at the respiratory and the gastrointestinal surfaces. The bacteria are eventually taken up by phagocytic cells lying underneath the submucosa. Previous studies have shown that macrophages ingest *Brucella* through 'zipper-like' phagocytosis [10]. Opsonized *Brucella* are internalized via complement or Fc receptors while non-opsonized smooth bacteria exploit lipid rafts and some other unidentified receptors to enter macrophages [11,12,13]. Mechanism of cell-invasion is linked to bacterial fate; opsonized *Brucella* employ lipid raft-independent entry and are preferentially degraded in macrophages [13].

Upon internalization, surviving bacteria are found within *Brucell*a-containing vacuoles (BCVs). The bacteria in BCV modulates the vesicle trafficking to reach endoplasmic reticulum (ER)-derived vacuole which is the replicative niche for *Brucella* (Fig. 1.1). During the first few minutes after cellular invasion, BCV undergoes a series of transient interactions with components of the endosomal pathway. Interaction with early endosomes is confirmed by the presence of early endosomal antigen 1 (EEA 1), transferrin receptor and small GTP binding protein Rab5 in the BCV (Fig. 1.1) [14]. BCVs also interact with late endosomes and lysosomes. These interactions are transient and occur prior to the interactions of BCV with ER membranes and the formation of a replication-permissive compartment. Presence of CD63, Rab7-interacting lysosomal protein (RILP), lysosomal-associated membrane protein 1 (LAMP-1) and small GTPase Rab7 in BCV is suggestive of its interaction with late endosomes/lysosomes (Fig. 1.1) [15]. At this point, unlike killed *Brucella*, live *Brucella* spp. limit interaction of their vacuoles with the lysosomes, possibly to avoid accumulating degradative contents to bactericidal levels [15]. These vacuole maturation steps take up to 12 hrs to be completed after which BCVs transform into ER-derived replicative organelles. Sustained interaction of BCVs with ER leads to the progressive loss of LAMP-1 and acquisition of ER-specific markers including calreticulin (Fig. 1.1) [14,16,17].

The formation of ER-derived replicative organelle depends on the interaction of BCV with specific components of the early secretory pathway [17]. The early secretory pathway is

initiated at ER-exit sites (ERES), where transport vesicle formation is mediated by the coat complex II (COPII). The organization and functionality of ERES domains are regulated by small GTPase Sar1 [18]. Subsequent protein transport requires the activity of ADP-ribosylation factor 1 (ARF1) which recruits components of COPI to the ER membranes [19]. A previous study by Celli et al. demonstrates that BCV interacts with ERES and disruption of ERES by inhibiting Sar1 activity impairs *Brucella* replication; however, inhibition of Arf1 activity had no effect on the ability of BCVs to mature into replicative organelles [17].

Virulence factors of *Brucella*

Persistent infection is an adaptation that *Brucella* needs to evade the host defense system until transmission can occur. *Brucella* spp. rely on several virulence factors that contribute to disarming the host macrophages and ensuring *Brucella* survival. Knowledge of these factors has significantly enhanced our understanding of how *Brucella* directs its intracellular cycle to avoid the formation of phagolysosomes and acquire a replication-permissive niche.

Lipopolysaccharide (LPS)

LPS of *Brucella* is composed of three components: lipid A, a core oligosaccharide and an O-polysaccharide (O-PS). Lipid A forms the hydrophobic anchor of the LPS while O-PS extends into the extracellular environment. LPS is one of the major virulence factors of *Brucella* [7]. Based on the LPS structure, *Brucella* spp. can be separated into smooth and rough strains. Smooth strains possess O-PS in their LPS moiety while strains that are devoid of O-PS exhibit a rough phenotype. O-PS of *Brucella* confers serum and complement resistance and it is also involved in protecting *Brucella* against microbicidal phagocytic conditions [7]. Consequently, smooth forms are generally more virulent than

their rough counterparts [20]. However, *B. canis* and *B. ovis* are rough species that are virulent in their natural hosts [20].

Genetics of *Brucella* O-PS biosynthesis

The biosynthetic pathway of smooth LPS of *Brucella* has not yet been fully characterized. However, several genes have been found to be essential for the synthesis of O-PS. These genes are located on two genetic loci, *wbk* and *wbo*, of *Brucella* chromosome I (Fig. 1.2) [21]. Region *wbo* contains two genes, *wboA* and *wboB*, encoding glycosyltransferases while region *wbk* contains genes involved in synthesis of perosamine (*gmd* and *per*), its formylation (*wbkC*) and polymerization (*wbkA* and *wbkE*), its translocation (*wzm* and *wzt*) as well as those for bactoprenol priming (*wbkD* and *wbkF*) (Fig. 1.2) [21]. *wbk* also contains genes encoding products that furnish the perosamine precursor, mannose [21]. *Brucella* O-PS is first assembled onto bactoprenol phosphate on the cytoplasmic face of inner bacterial membrane, following which it is translocated across the inner membrane. This translocation event is mediated by an ATP-binding cassette (ABC) transporter system [20]. Mutations in genes that are required for the synthesis of perosamine or bactoprenol-P-P-NAc-aminosugars also affect the synthesis of O-PS (Fig. 1.2) [20]. Using transposon mutagenesis, Gonzales et al. demonstrated that mutations in 13 genes (*wa***, *wbkE*, *wbkA*, *gmd*, *per*, *wzm*, *wbkF*, *wbkD*, *prm*, *ManBcore*, *wboA*, *wboB* and *manB*) conferred a rough phenotype to *B. melitensis* [20]. They also found that *wboA* and *wboB* genes are absent in *B. ovis* genome contributing to its rough phenotype [20,21]. *B. canis*, on the other hand, was found to have disruptions in *wbkF* (encodes undecaprenyl-glycosyltransferase) and *wbkD* (encodes epimerase/dehydratase) genes, both of which are required for O-PS synthesis [20,21].

Function of smooth LPS during *Brucella* infection

The LPS of *Brucella* is non-classical when compared with the classical LPS moiety of enterobacterial species. The endotoxin component of *Brucella* LPS, lipid A, has several distinct features that distinguish it from the lipid A of other Gram-negative bacteria.

Brucella lipid A contains a diaminoglucose backbone (rather than glucosamine) and long chain fatty acids C28 (instead of C18-C19) [22]. Due to the difference in lipid A composition, *Brucella* LPS is several hundred times less toxic than *E. coli* LPS [23]. It is a poor inducer of respiratory burst, reactive nitrogen intermediates as well as lysozyme [24,25]. Also, it is poorly recognized by the innate immune sensor Toll-like receptor (TLR) 4 and it does not induce the secretion of pro-inflammatory cytokines by infected host cells, thereby evading host immune responses [23]. The presence of long-chain fatty acids in lipid A also contributes to the reduced activity of *Brucella* LPS in inducing inflammatory responses [26]. Also, smooth *Brucella* LPS impairs host cells from activating *Brucella*-antigen-specific adaptive immune responses. It forms large clusters or macrodomains with MHC-II and interferes with antigen presentation to host $CD4^+$ T cells [27].

Smooth *Brucella* LPS also plays a role in lipid-raft mediated entry into host cells. *Brucella* strains lacking O-PS enter macrophages in a lipid-raft independent manner and are targeted to lysosomes for degradation [28]. The BCVs containing O-PS deficient strains fuse more rapidly with lysosomes when compared with BCVs containing heat-killed smooth *Brucella* [28], indicating that O-PS is essential for the intracellular trafficking of *Brucella*. It is possible that *Brucella* is able to exploit a survival-permissive entry only when its O-PS interacts with particular receptor(s) associated with lipid-rafts or alternatively, the O-PS might modify the fusogenic properties of the BCV membrane [29].

Brucella Type IV secretion system (T4SS)

Type IV secretion systems (T4SS) are membrane-associated transporter complexes which are closely related to bacterial conjugation systems. They are present in many Gram-negative bacteria including *Agrobacterium tumefaciens*, *Legionella pneumophila* and *Helicobacter pylori*. T4SSs mediate the secretion of multimeric protein toxins, monomeric proteins and nucleoprotein complexes. Hence, they are extremely versatile (unlike other secretion systems) in the choice of the secreted substrate and also the target-cell types, which can be bacteria (same/different species), fungi, plants or mammalian cells [30-32]. Apart from delivering a wide variety of substrates into the target host cells, T4SS can also

mediate DNA uptake and subsequent release into the extracellular milieu. Moreover, T4SS is involved in the conjugative transfer of plasmid DNA and transposons into a variety of bacterial species. This genetic exchange helps the bacteria to adapt to the changes in the host during infection while the delivery of effectors into target cells aids in suppressing the host defense system thereby allowing the bacteria to replicate and persist.

Architecture of *Brucella* T4SS

T4SS of *Brucella* is encoded by *virB* operon which consists of 12 genes: *virB1* to *virB12*. *Brucella* T4SS is not yet fully characterized; however, much has been inferred from the similarity of *Brucella* VirB proteins to components of T4SS in bacteria like *Agrobacterium tumefaciens* [8]. Proteins VirB8, VirB9 and VirB10 interact with each other to form the core structure that spans the outer and inner membrane of bacterium [8,33-35]. VirB6 and VirB7 might act to stabilize the core structure [36,37] while the ATPase, which consists of VirB4 and VirB11, provides energy for the assembly of the T4SS as well as for the delivery of effector proteins [38,39]. VirB2 and VirB5 form the pilus while VirB3 mediates the assembly of the pilus [40]. The effectors are translocated to the eukaryotic target cell through the pilus. Previous studies have shown that *virB1*, *virB7* and *virB12* are not essential for the enhanced persistence of *B. abortus* [41-43]. The encoded proteins VirB1, VirB7 and VirB12 do not contribute to the structure of the core complex or the pilus in any bacteria containing the T4SS and might be dispensable for the function of T4SS [43,44].

Function of T4SS during *Brucella* infection

Brucella strains lacking a functional T4SS are attenuated in macrophages as well as in laboratory and natural host animals [8,45,46]. *Brucella virB* mutants are incapable of preventing the fusion of phagosomes with lysosomes and are gradually cleared from the host [47]. Although phagosomes containing wild-type *Brucella* do transiently fuse with early and late endosomes and lysosomes, they restrict this interaction to avoid the formation of phagolysosomes and subsequent degradation [15]. This transient fusion with the components of the endosomal pathway leads to acidification of the *Brucella*-containing

phagosomes; acidification is essential for the induction of expression of the *virB* genes. It has been observed that wild-type *Brucella* are able to rescue the survival of *virB* mutants [48] indicating that T4SS-intact *Brucella* secrete effectors that are translocated into the cells containing *virB* mutants.

Regulation of *Brucella* T4SS

virB T4SS is induced in acidic pH and also in nutrient restricted conditions [49,50]. Three regulators are involved in the induction of T4SS under nutrient starvation. They include the stringent response regulator Rsh, integration host factor (IHF) and the histidine utilization regulator HutC [51,52]. Also, two LuxR family regulators, VjbR (Vacuolar jacking *Brucella* regulator) and BabR (*Brucella abortus* regulator), modulate the expression of *virB* genes [53,54]. Both VjbR and BabR control the expression of genes involved in virulence, stress response, metabolism and replication; however, they act in the opposite manner [54-56]. It is possible that the activity of these regulators helps *Brucella* to adapt in nutrient-limitied conditions after host cell infection until the formation of an ER-derived replication-permissive vacuole [14]. At this stage, VjbR is turned off while BabR is activated [55]. BabR represses T4SS activity and enables *Brucella* to replicate in the ER-derived vacuole [14].

Effectors translocated by *Brucella* T4SS

Unlike *Bartonella*, the genes encoding the effector molecules in *Brucella* are not co-located with the *virB* genes [57]. Thus, identification of effector proteins translocated through the T4SS in *Brucella* has been a formidable task. Till date, only a few effector proteins have been identified.

The first two T4SS effectors identified were *virB*-coregulated effectors A and C (VceA and VceC) [58]. These effectors were identified in a screen for VjbR-activated promoters of genes that are co-regulated with *virB* genes. Translocation of VceA and VceC was found to be dependent on VirB as demonstrated by generating fusions of the effectors to TEM-1 beta-lactamase. Translocation of these fusion proteins was detected starting at 7-9 hrs post-

infection which is consistent with the time of phagosomal acidification and induction of T4SS. *Legionella pneumophila* Dot/Icm system was also able to translocate *B. suis* VceC indicating that the secretion mechanisms are conserved to some extent [58]. A more recent study has shown that VceC induces ER stress and secretion of proinflammatory cytokines [59].

Translocation of four other effector proteins (BPE123, BPE005, BPE275 and BPE043) was found to be dependent on VirB T4SS [60]. They were identified by screening for proteins for eukaryotic-like domains or domains that participate in protein-protein interactions [60]. Their intracellular functions are still unknown. Also, Rab2-interacting conserved protein (RicA), another effector translocated by T4SS, was identified by a yeast two-hybrid screening assay for *Brucella* proteins that potentially interact with host proteins [61]. RicA binds preferentially to GDP-bound Rab2 and is involved in recruiting Rab2 GTPase to the *Brucella*-containing phagosome. The recruitment of Rab2 to BCV is considered crucial for the sustained interactions of *Brucella* with ER by intercepting secretory vesicles [61]. Hence, Rab2 is essential for the intracellular replication and trafficking of *Brucella* [62]. However, *ricA* deletion mutant is not attenuated in virulence even though blocking Rab2 leads to inhibition of intracellular replication of *Brucella* [61]. This indicates that several additional proteins might play a redundant role in the serial recruitment of eukaryotic factors during the maturation of the BCV.

More recently, the translocation of 5 effector proteins (BspA, BspB, BspC, BspE, BspF) has been demonstrated to be dependent on VirB T4SS [63]. Among them, BspA, BspB and BspF inhibit the host protein secretion by impairing the vesicular cargo transport from ER to the Golgi apparatus [63]. Mutation in these three genes (*bspA*, *bspB* and *bspF*) affect the ability to replicate and persist within host cells [63].

Quorum sensing in Brucella

Quorum sensing (QS) is the regulation of gene expression in response to small diffusible chemical signaling molecules, autoinducers, which are produced and secreted by bacteria. These autoinducers increase in concentration in response to changes in cell-population

density. The detection of a minimal threshold concentration of these signaling molecules leads to modulation in gene expression. This sensing system is used both by Gram-positive as well as Gram-negative bacteria to regulate diverse processes including virulence, biofilm formation, conjugation, and antibiotic production etc. In general, Gram-negative bacteria use N-acyl homoserine lactone (AHL) to communicate, and Gram-positive bacteria use processed oligo-peptides as autoinducing molecules [64]. The ability of the bacteria to communicate with each other allows them to coordinate gene expression and therefore the behavior of the overall population.

AHL based QS requires an AHL synthase and a target transcriptional regulator that can recognize the AHL molecules and alter the expression of target genes. The autoinducer in *Brucella* was identified to be N-dodecanoylhomoserine lactone (C_{12}-HSL) in the supernatants of *B. melitensis* culture [56,65]. However, the concentration of C_{12}-HSL in *Brucella* culture supernatant was found to be low. It might be that the *in vivo* AHL production is much higher than *in vitro* production, or alternatively, the hydrophobic long-chain AHLs interact with the *Brucella* membrane leading to a lowered concentration in the culture supernatant. Attempts to identify an AHL synthase in *Brucella* have not yielded any promising results.

Two bonafide QS regulators in *Brucella* are VjbR and BabR [53,54]. They both contain an AHL binding domain as well as a C-terminal HTH (helix turn helix) DNA binding domain. *vjbR* mutants are attenuated and unable to reach their ER-derived replicative vacuole [14]. As discussed above, VjbR is required for *virB* expression. Previous experiments have demonstrated that addition of exogenous C_{12}-HSL impairs *Brucella* trafficking to its replicative vacuole. Also, C_{12}-HSL decreases the production of VirB8 by repressing the transcription from *virB* promoter. C_{12}-HSL-mediated repression of *virB* genes is through its inhibitory effect on VjbR [55,56]. In contrast, C_{12}-HSL has an activating effect on BabR [55,56]. Previous transcriptomic studies and qRT-PCR analysis suggest that there is cross-talk between VjbR and BabR; significant overlap exists between VjbR- and BabR-regulated genes [55,56]. However, comparative studies indicate that the loss of VjbR has a more pronounced effect on *Brucella* virulence when compared to the loss of BabR suggesting that both these regulators are not functionally redundant [54].

The two-component BvrR/BvrS system in Brucella

Most prokaryotic signal transduction units consist of two components: a histidine kinase that senses the environmental alterations and a response regulator protein. The histidine protein kinase autophosphorylates at a conserved histidine residue creating a high energy phosphoryl group that subsequently phosphorylates a conserved aspartate residue in the response regulator protein. This phosphorylation induces activation of factors that interact with specific DNA sequences to affect gene expression. In this way, two-component systems control the response of bacteria to a number of stimuli that regulate important cell functions including response to stress and virulence [66].

BvrR/BvrS (Bvr: *Brucella* virulence related) is one of the best characterized two-component system in *Brucella*. Mutants in this system are highly attenuated and sensitive to the bactericidal action of polycations including polymyxin and also to non-immune human and cattle sera. The *bvrS* mutant is unable to recruit the small GTPases that are required for polymerization of actin and subsequent cell invasion [67]. The BvrR/BvrS system also regulates intracellular trafficking of *Brucella* as the mutant strains are incapable of replicating in macrophages and epithelial cells; they are unable to avoid the formation of phagolysosomes and are cleared from the host in less than 12 days [68].

Numerous genes are subject to BvrR/BvrS regulation: some examples include genes encoding specific outer membrane proteins (Omp3a and Omp3b), lipoproteins, glycosyltransferases, ABC-type transport system, genes required for biosynthesis of fatty acids and flagellum, genes involved in the Krebs cycle and metabolism of amino acids, fatty acids and nitrogen [9,69]. All these genes are differentially expressed in the *bvrR* mutant. Also, seven transcriptional regulators including VjbR are differentially regulated in the *bvrR* mutant. BvrR/BvrS system regulates the expression of VirB at the transcriptional level and also directly controls the *vjbR* expression [70]. Hence, BvrR/BvrS is a master system that interacts with other regulators to alter the expression of genes involved in synthesis of the cell envelope, metabolism and virulence-related genes and potentially benefits *Brucella* spp. in successful adaptation to their intracellular lifestyle.

Other virulence factors that contribute to Brucella survival and replication

Brucella resistance to the microbicidal activity of phagosomes

Several virulence factors of *Brucella* are involved in enhancing the resistance to the microbicidal host defenses. Microbicidal function of professional phagocytes relies in part on the reactive oxygen species (ROS) which are generated either directly or indirectly by NOX2 NADPH oxidase. Superoxide is released within the phagosomal lumen when the active oxidase transfers the electrons from cytosolic NADPH to O_2 [71]. Dismutation of superoxide can lead to the formation of H_2O_2 which in turn reacts with superoxide to generate hydroxyl radicals and singlet oxygen [72]. Similar to ROS, reactive nitrogen species (RNS) are also important microbicidal effectors. Within the phagosomal compartment, nitric oxide can undergo a spontaneous or catalytic conversion into RNS such as nitrogen dioxide, peroxynitrite, dinitrogen trioxide and nitrosothiols [73]. These RNS and ROS can interact with a variety of microbial targets like nucleic acids, protein tyrosine residues, thiols, and lipids [74] ultimately resulting in inhibition of microbial replication.

Phagocytes also secrete a set of proteins that either prevent the growth of the pathogen or compromise the microbial membrane integrity. For example, scavengers can directly limit the availability of essential nutrients to prevent microbial growth. Also, phagosomal membranes can acquire various transporters. As an example, natural resistance-associated macrophage protein 1 (NRAMP1) can be recruited to the phagosomal membrane from late endosomes and lysosomes. NRAMP1 exerts a bacteriostatic effect on pathogens by exclusive extrusion of divalent cations from the phagosomal lumen [75]. Phagosomes can also disrupt the integrity of pathogens by the action of defensins, lysozymes, proteases and lipases. Defensins induce membrane permeabilization of bacteria by binding to negatively charged molecules on the pathogen surface and forming ion-permeable channels [76].

Despite the presence of these microbicidal host factors, *Brucella* spp. can survive inside host cells. They have evolved various strategies to counteract host defenses. *Brucella* spp. possess enzymes that can either directly detoxify the ROS or repair the oxidative damage

to cellular components. Among them, superoxide dismutases (SODs) are a family of metalloenzymes that can dismutate superoxide into H_2O_2 and O_2 and protect *Brucella* from the oxidative burst of macrophages [77]. Two enzymes, catalase (encoded by *katA*) and the alkyl hydroperoxide reductase (encoded by *ahpC* and *ahpD*) have a role in protection from H_2O_2 [78,79]. Peroxiredoxin AhpC scavenges endogenous H_2O_2 that is generated by aerobic respiration while KatE detoxifies the supraphysiologic levels of H_2O_2 [78,79]. *Brucella* can also avoid oxidative damage indirectly. Cytochrome *bd* ubiquinol oxidase and *cbb3*-type cytochrome c oxidases prevent ROS toxicity by scavenging O_2 [80]. They are involved in adaptation of *Brucella* to oxygen-limiting conditions [80]. *Brucella* also expresses exonuclease III, encoded by *xthA*, which removes the oxidative lesions from the bacterial DNA [81]. This base excision repair system has been found to protect *Brucella* from oxidative damage *in vitro* [81]. For resistance to nitrosative damage, *Brucella* expresses nitric oxide reductase (*norD*) which detoxifies NO and enhances intracellular bacterial survival [82].

Brucella is inherently more resistant to the action of microbicidal cationic peptides due to the structure of its non-canonical LPS. *Brucella* LPS is a poor inducer of respiratory burst, reactive nitrogen intermediates as well as lysozyme (as discussed earlier) [24,25]. It also protects the bacteria against a variety of cationic peptides such as defensins, lactoferrin, lysozyme, bactenecin-derived peptides [83]. This resistance is partly due to the reduced number of phosphate groups in the lipid A moiety which facilitate a close aggregation of LPS molecules via their hydrophobic fatty acids, thereby reducing the penetration by cationic peptides [7,83,84].

Following entry in phagocytes, *Brucella* needs to adapt under nutrient-deprived intracellular environment to survive. This physiological adaptation is in part facilitated by the stringent response mediator Rsh [52]. Rsh deletion mutants exhibit altered cellular morphology and reduced survival under starvation conditions *in vitro* and *in vivo* [52]. Also, Rsh participates indirectly in the VirB-mediated formation of the ER-derived replicative niche for *Brucella* [52]. Under iron-deprived conditions, *Brucella* is capable of scavenging and storing iron. There is a link between the production of siderophores and

the survival of *Brucella* in the presence of erythritol under iron-limiting conditions *in vitro* [85]; however, the role of siderophores in virulence is not clearly understood.

Brucella spp. also inhibit apoptosis of the infected host macrophages [86] which is likely a strategy exploited by *Brucella* to survive intracellularly and evade host adaptive responses. The precise mechanism by which *Brucella* spp. inhibit host cell apoptosis is not clearly understood; previous studies provide valuable insights into possible mechanisms for inhibition of apoptosis. *Brucella* downregulates mitochondrion-associated proapoptotic genes [87] and upregulates the expression of anti-apoptotic factors [86]. Further, a recent study shows that *Brucella* infection results in calcium-dependent degradation of the protease calpain2, and subsequent prevention of caspase 3 (apoptotic effector) activation [88]. *Brucella* mutants lacking the O-PS fail to inhibit apoptosis [89] indicating that O-PS of *Brucella* plays a crucial role in infection-induced inhibition of apoptosis.

Table 1.1 briefly summarizes the virulence factors of *Brucella* and their roles in enhancing the survival of *Brucella*.

Concluding remarks

Brucella spp. employ several strategies to deceive the host and survive in host cells for a considerable time. This is important as *Brucella* spp. have to persist within their hosts until transmission occurs which in natural hosts is primarily via aborted fetal tissues, sexual contact and shedding in milk. As some mammalian hosts of *Brucella* can only breed once or twice annually, the capacity of *Brucella* to successfully survive intracellularly is critical to their virulence [44]. Following cell invasion, *Brucella* spp. avoid the induction of an aggressive inflammatory response and subsequently, regulate the trafficking of their membrane-bound vacuole so as to avoid the fusion with lysosomes. Several virulence factors of *Brucella* participate to promote its intracellular trafficking and persistence within the host. *Brucella* also exploits some host defense mechanisms such as acidic pH and nutrient limitation for the induction of genes that modulate its intracellular cycle. Recent developments including the identification of the T4SS effector molecules and their

functional characterization would further enhance our understanding of the molecular complexity of *Brucella* adaptation during its coevolution with host animals.

References

[1] Meador, V. P., Deyoe, B. L., 1989. Intracellular localization of *Brucella abortus* in bovine placenta. Vet Pathol 26, 513–515.

[2] O'Callaghan, D., Whatmore, A. M., 2011. *Brucella* genomics as we enter the multi-genome era. Brief Funct Genomics 10, 334–341.

[3] Scholz, H. C., Nockler, K., Gollner, C., Bahn, P., Vergnaud, G., Tomaso, H., Al Dahouk, S., Kampfer, P., Cloeckaert, A., Maquart, M., Zygmunt, M. S., Whatmore, A. M., Pfeffer, M., Huber, B., Busse, H., De, B. K., 2010. *Brucella inopinata* sp. nov., isolated from a breast implant infection. Int J Syst Evol Microbiol 60, 801–808.

[4] Tiller, R. V., Gee, J. E., Frace, M. A., Taylor, T. K., Setubal, J. C., Hoffmaster, A. R., De, B. K., 2010a. Characterization of novel *Brucella* strains originating from wild native rodent species in North Queensland, Australia. Appl Environ Microbiol 76, 5837–5845.

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

[6] Moreno, E., Moriyo´n, I., 2001. The genus *Brucella*. In: The prokaryotes. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K. H., Stackebrandt, E. (Eds.), Electronic Version, Springer, New York.

[7] Lapaque, N., Moriyon, I., Moreno, E., Gorvel, J. P., 2005. *Brucella* lipopolysaccharide acts as a virulence factor. Curr Opin Microbiol 8, 60-66.

[8] O'Callaghan, D., Cazevieille, C., Allardet-Servent, A., Boschiroli, M. L., Bourg, G., Foulongne, V., Frutos, P., Kulakov, Y., Ramuz, M., 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol Microbiol 33, 1210-1220.

[9] Guzman-Verri, C., Manterola, L., Sola-Landa, A., Parra, A., Cloeckaert, A., Garin, J., Gorvel, J. P., Moriyon, I., Moreno, E., Lopez-Goni, I., 2002. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the Rhizobiaceae. Proc Natl Acad Sci USA 99, 12375-12380.

[10] Ackermann, M. R., Cheville, N. F., Deyoe, B. L., 1988. Bovine ileal dome lymphoepithelial cells: endocytosis and transport of *Brucella abortus* strain 19. Vet Pathol 25, 28–35.

[11] Campbell, G. A., Adams, L. G., Sowa, B. A., 1994. Mechanism of binding of *Brucella abortus* to mononuclear phagocytes from cows naturally resistant or susceptible to brucellosis. Vet Immunol Immunopathol 41, 295–306.

[12] Harmon, B. G., Adams, L. G., Frey, M., 1988. Survival of rough and smooth strains of *Brucella abortus* in bovine mammary gland macrophages. Am J Vet Res 49, 1092–1097.

[13] Watarai, M., Makino, S., Fujii, Y., Okamoto, K., Shirahata, T., 2002. Modulation of *Brucella*-induced micropinocytosis by lipid rafts mediates intracellular replication. Cell Microbiol 4, 341-355.

[14] Celli, J., de Chastellier, C., Franchini, D. M., Pizarro-Cerda, J., Moreno, E., Gorvel, J. P., 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J Exp Med 198, 545-556.

[15] Starr, T., Ng, T. W., Wehrly, T. D., Knodler, L. A., Celli, J., 2008. *Brucella* intracellular replication requires trafficking through the late endosomal/lysosomal compartment. Traffic 9, 678-694.

[16] Pizarro-Cerda, J., Meresse, S., Parton, R. G., van der Goot, G., Sola-Landa, A., Lopez-Goni, I., Moreno, E., Gorvel, J. P., 1998. *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infect Immun 66, 5711-5724.

[17] Celli, J., Salcedo, S. P., Gorvel, J. P., 2005. *Brucella* coopts the small GTPase Sar 1 for intracellular replication. Proc Natl Acad Sci USA 102, 1673-1678.

[18] Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M., Schekman, R., 1994. COPII: A membrane coat formed by set proteins that drive vesicle budding from the endoplasmic reticulum. Cell 77, 895-907.

[19] Scales, S. J., Pepperkok, R., Kreis, T. E., 1997. Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. Cell 90, 1137-1148.

[20] Gonzalez, D., Grillo, M. J., de Miguel, M. J., Ali, T., Arce-Gorvel, V., Delrue, R. M., Conde-Alvarez, R., Munoz, P., Lopez-Goni, I., Iriarte, M., Marin, C. M., Weintraub, A., Widmalm, G., Zygmunt, M., Letesson, J. J., Gorvel, J. P., Blasco, J. M., Moriyon, I., 2008. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLoS One 3, e2760.

[21] Zygmunt, M. S., Blasco, J. M., Letesson, J. J., Cloeckaert, A., Moriyon, I., 2009. DNA polymorphism analysis of *Brucella* lipopolysaccharide genes reveals marked differences in O polysaccharide biosynthetic genes between smooth and rough *Brucella* species and novel species-specific markers. BMC Microbiology 9, 92-105.

[22] Moriyon, I., 2003. Against Gram-negative bacteria: the LPS case. In: Intracellular pathogens in membrane interactions and vacuole biogenesis. Gorvel, J. P. (Ed.), Georgestown, Texas: Landes Bioscience/Eurekah.com, 204-230.

[23] Goldstein, J., Hoffman, T., Frasch, C., Lizzio, E. F., Beining, P. R., Hochstein, D., Lee, Y. L., Angus, R. D., Golding, B., 1992. Lipopolysaccharide (LPS) from *Brucella abortus* is less toxic than that from *Escherichia coli*, suggesting the possible use of *B. abortus* or LPS from *B. abortus* as a carrier in vaccines. Infect Immun 60, 1385-1389.

[24] Moreno, E., Berman, D. T., Boettcher, L. A., 1981. Biological activities of *Brucella abortus* lipopolysaccharides. Infect Immun 31, 362-370.

[25] Rasool, O., Freer, E., Moreno, E., Jarstrand, C., 1992. Effect of *Brucella abortus* lipopolysaccharide on oxidative metabolism and lysozyme release by human neutrophils. Infect Immun 60, 1699-1702.

[26] Parent, M. A., Goenka, R., Murphy, E., LeVier, K., Carreiro, N., Golding, B., Fergusson, G., Roop, R. M. II, Walker, G. C., Baldwin, C. L., 2007. *Brucella bacA* mutant induces greater pro-inflammatory cytokines than the wild-type parent. Microbes Infect 9, 55-62.

[27] Forestier, C., Moreno, E., Meresse, S., Phalipon, A., Olive, D., Sansonetti, P., Gorvel, J. P., 1999. Interaction of *Brucella abortus* lipopolysaccharide with major histocompatibility complex class II molecules in B lymphocytes. Infect Immun 67, 4048-4054.

[28] Porte, F., Naroeni, A., Ouahrani-Bettache, S., Liautard, J. P., 2003. Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome fusion in murine macrophages. Infect Immun 71, 1481-1490.

[29] Celli, J., 2006. Surviving inside a macrophage: The many ways of *Brucella*. Res Microbiol 157, 93-98.

[30] Bundock, P., den Dulk Ras, A., Beijersbergen, A., Hooykaas, P. J., 1995. Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J 14, 3206–3214.

[31] Zhu, J., Oger, P. M., Schrammeijer, B., Hooykaas, P. J. J., Farrand, S. K., Winans, S. C., 2000. The bases of crown gall tumorigenesis. J Bacteriol 182, 3885–3895.

[32] Waters, V. L., 2001. Conjugation between bacterial and mammalian cells. Nature Genet 29, 375–376.

[33] Chandran, V., Fronzes, R., Duquerroy, S., Cronin, N., Navaza, J., Waksman, G., 2009. Structure of the outer membrane complex of a type IV secretion system. Nature 462, 1011-1015.

[34] Fronzes, R., Schafer, E., Wang, L., Saibil, H. R., Orlova, E. V., Waksman, G., 2009. Structure of a type IV secretion system core complex. Science 323, 266-268.

[35] Sivanesan, D., Hancock, M. A., Villamil Giraldo, A. M., Baron, C., 2010. Quantitative analysis of VirB8-VirB9-VirB10 interactions provides a dynamic model of type IV secretion system core complex assembly. Biochemistry 49, 4483-4493.

[36] Baron, C., Thorstenson, Y. R., Zambryski, P. C., 1997. The lipoprotein VirB7 interacts with VirB9 in the membranes of *Agrobacterium tumefaciens*. J Bacteriol 179, 1211-1218.

[37] Jakubowski, S. J., Krishnamoorthy, V., Christie, P. J., 2003 *Agrobacterium tumefaciens* VirB6 protein participates in formation of VirB7 and VirB9 complexes required for type IV secretion. J Bacteriol 185, 2867-2878.

[38] Watarai, M., Makino, S., Shirahata, T., 2002. An essential virulence protein of *Brucella abortus*, VirB4, requires an intact nucleoside-triphosphate-binding domain. Microbiology 148, 1439-1446.

[39] Alvarez-Martinez, C. E, Christie, P. J., 2009. Biological diversity of prokaryotic type IV secretion systems. Microbiol Mol Biol Rev 73, 775-808.

[40] Schmidt-Eisenlohr, H., Domke, N., Angerer, C., Wanner, G., Zambryski, P. C., Baron, C., 1999. Vir proteins stabilize VirB5 and mediate its association with the T pilus of *Agrobacterium tumefaciens*. J Bacteriol 181, 7485-7492.

[41] Den Hartigh, A. B., Sun, Y. H., Sondervan, D., Heuvelmans, N., Reinders, M. O., Ficht, T. A., Tsolis, R. M., 2004. Differential requirements for VirB1 and VirB2 during *Brucella abortus* infection. Infect Immun 72, 5143-5149.

[42] Den Hartigh, A. B., Rolan, H. G., de Jong, M. F., Tsolis, R. M., 2008. VirB3 to VirB6 and VirB8 to VirB11, but not VirB7, are essential for mediating persistence *Brucella* in the reticuloendothelial system. J Bacteriol 190, 4427-4436.

[43] Sun, Y. H., Rolan, H. G, Den Hartigh, A. B., Sondervan, D., Tsolis, R. M., 2005. *Brucella abortus virB12* is expressed during infection but is not an essential component of the type IV secretion system. Infect Immun 73, 6048-6054.

[44] de Jong, M. F., Tsolis, R. M., 2012. Brucellosis and type IV secretion system. Fut Microbiol 7, 47-58.

[45] Sieira, R., Comerci, D. J., Sanchez, D. O., Ugalde, R. A., 2000. A homologue of an operon required for DNA transfer in *Agrobacterium* is required in *Brucella abortus* for virulence and intracellular multiplication. J Bacteriol 182, 4849-4855.

[46] Zygmunt, M. S., Hagius, S. D., Walker, J. V., Elzer, P. H., 2006. Identification of *Brucella melitensis* 16M genes required for bacterial survival in the caprine host. Microbes Infect 8, 2849-2854.

[47] Rolan, H. G., Tsolis, R. M., 2007. Mice lacking components of adaptive immunity show increased *Brucella abortus virB* mutant colonization. Infect Immun 75, 2965-2973.

[48] Nijskens, C., Copin, R., De Bolle, X., Letesson, J. J., 2008. Intracellular rescuing of a *B. melitensis* 16M *virB* mutant by co-infection with a wild type strain. Microb Pathog 45, 134-141.

[49] Boschiroli, M., Ouahrani-Bettache, S., Foulongne, V., Michaux-Charachon, S., Bourg, G., Allardet-Servent, A., Cazevieille, C., Liautard, J. P., Ramuz, M., O'Callaghan, D., 2002. The *Brucella suis virB* operon is induced intracellularly in macrophages. Proc Natl Acad Sci USA 99, 1544-1549.

[50] Rouot, B., Alvarez-Martinez, M. T., Marius, C.,Menanteau, P., Guilloteau, L., Boigegrain, R. A., Zumbihl, R., O'Callaghan, D., Domke, N., Baron, C., 2003. Production of the type IV secretion system differs among *Brucella* species as revealed with VirB5- and VirB8-specific antisera. Infect Immun 71, 1075-1082.

[51] Sieira, R., Comerci, D. J., Pietrasanta, L. I., Ugalde, R. A., 2004. Integration host factor is involved in transcriptional regulation of the *Brucella abortus virB* operon. Mol Microbiol 54, 802-822.

[52] Dozot, M., Boigegrain, R. A., Delrue, R. M., Hallez, R., Ouahrani-Bettache, S., Danese, I., Letesson, J. J., De Bolle, X., Kohler, S., 2006. The stringent response mediator Rsh is required for *Brucella melitensis* and *Brucella suis* virulence, and for expression of the type IV secretion system *virB*. Cell Microbiol 8, 1791-1802.

[53] Delrue, R. M., Deschamps, C., Leonard, S., Nijskens, C., Danese, I., Schaus, J. M., Bonnot, S., Ferooz, J., Tibor, A., De Bolle, X., Letesson, J. J., 2005. A quorum-sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of *Brucella melitensis.* Cell Microbiol 7, 1151-1161.

[54] Rambow-Larsen, A. A., Rajashekara, G., Petersen, E., Splitter, G., 2008. Putative quorum-sensing regulator BlxR of *Brucella melitensis* regulates virulence factors including the type IV secretion system and flagella. J Bacteriol 190, 3274-3282.

[55] Uzureau, S., Lemaire, J., Delaive, E., Dieu, M., Gaigneaux, A., Raes, M., De Bolle, X., Letesson, J. J., 2010. Global analysis of quorum sensing targets in the intracellular pathogen *Brucella melitensis* 16M. J Proteome Res 9, 3200-3217.

[56] Weeks, J. N., Galindo, C. L., Drake, K. L., Adams, G. L., Garner, H. R., Ficht, T. A., 2010. *Brucella melitensis* VjbR and C12-HSL regulons: contributions of the N-dodecanoyl homoserine lactone signaling molecule and LuxR homologue VjbR to gene expression. BMC Microbiol 10, 167.

[57] Schulein, R., Guye, P., Rhomberg, T. A., Schmid, M. C., Schroder, G., Vergunst, A. C., Carena, I., Dehio, C., 2005. A bipartite signal mediates the transfer of type IV secretion substrates of *Bartonella henselae* into human cells. Proc Natl Acad Sci USA 102, 856-861.

[58] de Jong, M. F., Sun, Y. H., Den Hartigh, A. B., Van Dijl, J. M., Tsolis, R. M., 2008. Identification of VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the *Brucella* type IV secretion system. Mol Microbiol 70, 1378-1396.

[59] de Jong, M. F., Starr, T., Winter, M. G., den Hartigh, A. B., Child, R., Knodler, L. A., van Dijl, J. M., Celli, J., Tsolis, R. M., 2013. Sensing of bacterial type IV secretion via the unfolded protein response. MBio 4, e00418–12.

[60] Marchesini, M. I., Herrmann, C. K., Salcedo, S. P., Gorvel, J. P., Comerci, D. J., 2011. In search of *Brucella abortus* type IV secretion substrates: screening and identification of four proteins translocated into host cells through VirB system. Cell Microbiol 13, 1261-1274.

[61] De Barsy, M., Jamet, A., Filopon, D., Nicolas, C., Laloux, G., Rual, J. F., Muller, A., Twizere, J. C., Nkengfac, B., Vandenhaute, J., Hill, D. E., Salcedo, S. P., Gorvel, J. P., Letesson, J. J., De Bolle, X., 2011. Identification of a *Brucella* spp. secreted effector specifically interacting with human small GTPase Rab2. Cell Microbiol 13, 1044-1058.

[62] Fugier, E., Salcedo, S. P., De Chastellier, C., Pophillat, M., Muller, A., Arce-Gorvel, V., Fourquet, P., Gorvel, J. P., 2009. The glyceraldehyde-3-phosphate dehydrogenase and the small GTPase Rab 2 are crucial for *Brucella* replication. PLoS Pathog 5, e1000487.

[63] Myeni, S., Child, R., Ng, T. W., Kupko, J. J., Wehrly, T. D., Porcella, S. F., Knodler, L. A., Celli, J., 2001. *Brucella* modulates secretory trafficking via multiple Type IV secretion effector proteins. PLoS Pathog 9, e1003556.

[64] Miller, M. B., Bassler, B. L., 2002. Quorum sensing in bacteria. Annu Rev Microbiol 55, 165-199.

[65] Taminiau, B., Daykin, M., Swift, S., Boschiroli, M., Tibor, A., Lestrate, P., De Bolle, X., O'Callaghan, D., Williams, P., Letesson, J. J., 2002. Identification of a quorum-sensing signal molecule in the facultative intracellular pathogen *Brucella melitensis*. Infect Immun 70, 3004-3011.

[66] Stock, A. M., Robinson, V. L., Goudreau, P. N., 2000. Two-component signal transduction. Ann Rev Biochem 69, 183-215.

[67] Guzman-Verri, C., Chaves-Olarte, E., von Eichel-Streiber, C., Lopez-Goni, I., Thelestam, M., Arvidson, S., Gorvel, J. P., Moreno, E., 2001. GTPases of the Rho subfamily are required for *Brucella abortus* internalization in nonprofessional phagocytes: direct activation of Cdc42. J Biol Chem 276, 44435-44443.

[68] Sola-Landa, A., Pizarro-Cerda, J., Grillo, M. J., Moreno, E., Moriyon, I., Blasco, J. M., Gorvel, J. P., Lopez-Goni, I., 1998. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. Mol Microbiol 29, 125-138.

[69] Dozot, M., Poncet, S., Nicolas, C., Copin, R., Bouraoui, H., Maze, A., Deutscher, J., De Bolle, X., Letesson, J. J., 2010. Functional characterization of the incomplete phosphotransferase system (PTS) of the intracellular pathogen *Brucella melitensis*. PLoS One 5, e12679.

[70] Martinez-Nunez, C., Altamirano-Silva, P., Alvarado-Guillen, F., Moreno, E., Guzman-Verri, C., Chaves-Olarte, E., 2010. The two component system BvrR/BvrS regulates the expression of the Type IV secretion system VirB in *Brucella abortus*. J Bacteriol, 192, 5603-5608.

[71] Quinn, M. T., Gauss, K. A., 2004. Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases. J Leukoc Biol 76, 760-781.

[72] Minakami, R., Sumimotoa, H., 2006. Phagocytosis-coupled activation of the superoxide-producing phagocyte oxidase, a member of the NADPH oxidase (*nox*) family. Int J Hematol 84, 193-198.

[73] Fang, F. C., 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat Rev Microbiol 2, 820-832.

[74] De Groote, M. A., Fang, F. G., 1999. Nitric oxide and infection. Kluwer Academic-Plenum, New York.

[75] Cellier, M. F., Courville, P., Campion, C., 2007. Nramp1 phagocyte intracellular metal withdrawal defense. Microbes Infect 9, 1662-1670.

[76] Lehrer, R. I., Lichtenstein, A. K., Ganz, T., 1993. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu Rev Immunol 11, 105-128.

[77] Gee, J. M., Valderas, M. W., Kovach, M. E., Grippe, V. K., Robertson, G. T., Ng, W. L., Richardson, J. M., Winkler, M. E., Roop 2nd, R. M., 2005. The *Brucella abortus* Cu, Zn superoxide dismutase is required for optimal resistance to oxidative killing by murine macrophages and wild-type virulence in experimentally infected mice. Infect Immun 73, 2873–2880.

[78] Gee, J. M., Kovach, M. E., Grippe, V. K., Hagius, S., Walker, J. V., Elzer, P. H., Roop 2nd, R. M., 2004. Role of catalase in the virulence of *Brucella melitensis* in pregnant goats. Vet Microbiol 102, 111–115.

[79] Roop, R. M. II, Gee, J. M., Robertson, G. T., Richardson, J. M., Ng, W. L., Winkler, M. E., 2003. *Brucella* stationary-phase gene expression and virulence. Annu Rev Microbiol 57, 57–76.

[80] Endley, S., McMurray, D., Ficht, T. A., 2001. Interruption of the *cydB* locus in *Brucella abortus* attenuates intracellular survival and virulence in the mouse model of infection. J Bacteriol 183, 2454–2462.

[81] Hornback, M. L., Roop 2nd, R. M., 2006. The *Brucella abortus xthA*-1 gene product participates in base excision repair and resistance to oxidative killing but is not required for wild-type virulence in the mouse model. J Bacteriol 188, 1295–1300.

[82] Loisel-Meyer, S., Jimenez de Bagues, M. P., Basseres, E., Dornand, J., Kohler, S., Liautard, J. P., Jubier-Maurin, V., 2006. Requirement of *norD* for *Brucella suis* virulence in a murine model of *in vitro* and *in vivo* infection. Infect Immun 74, 1973–1976.

[83] Martinex de Tejada, G., Pizarro-Cerda, J., Moreno, E., Moriyon, I., 1995. The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. Infect Immun 63, 3054-3061.

[84] Velasco, J., Bengoechea, J., A., Brandenburg, K., Lindner, B., Seydel, U., Gonzalez, D., Zahringer, U., Moreno, E., Moriyon, I., 2000. *Brucella abortus* and its closest phylogenetic relative, *Ochrobactrum* spp., differ in outer membrane permeability and cationic peptide resistance.

[85] Bellaire, B. H., Elzer, P. H., Baldwin, C. L., Roop, R. M. II., 2003. Production of the siderophore 2, 3-dihydroxybenzoic acid is required for wild-type growth of *Brucella abortus* in the presence of erythritol under low-iron conditions *in vitro*. Infect Immun 71, 2927-2932.

[86] Gross, A., Terraza, A., Ouahrani-Bettache, S., Liautard, J. P., Dornand, J., 2000. In vitro *Brucella suis* infection prevents the programmed cell dealth of human monocytic cells. Infect Immun 68, 342-351.

[87] He, Y., Reichow, S., Ramamoorthy, S., Ding, X., Lathigra, R., Craig, J. C., Sobral, B. W. S., Schurig, G. G., Sriranganathan, N., Boyle, S. M., 2006. *Brucella melitensis* triggers time-dependent modulation of apoptosis and down-regulation of mitochondrion-associated gene expression in mice. Infect Immun 74, 5035-5046.

[88] Cui, G., Wei, P., Zhao, Y., Guan, Z., Yang, L., Sun, W., Wang, S., Peng, Q., 2014. *Brucella* infection inhibits macrophages apoptosis via Nedd4-dependent degradation of calpain 2.

[89] Fernandez-Prada, C. M., Zelazowska, E. B., Nikolich, M., Hadfield, T. L., Roop, R. M. II., Robertson, G. L., Hoover, D. L., 2003. Interactions between *Brucella melitensis* and human phagocytes: bacterial surface O-polysaccharide inhibits phagocytosis, bacterial killing and subsequent host cell apoptosis. Infect Immun 71, 2110-2119.

[90] Lavigne, J. P., Patey, G., Sangari, F. J., Bourg, G., Ramuz, M., O'Callaghan, D., Michaux-Charachon, S., 2005. Identification of a new virulence factor, BvfA, in *Brucella suis*. Infect Immun 73, 5524–5529.

[91] Arellano-Reynoso, B., Lapaque, N., Salcedo, S., Briones, G., Ciocchini, A. E., Ugalde, R., Moreno, E., Moriyon, I., Gorvel, J. P., 2005. Cyclic beta-1,2-glucan is a *Brucella* virulence factor required for intracellular survival. Nat Immunol 6, 618–625.

[92] Sangari, F. J., Seoane, A., Rodriguez, M. C., Aguero, J., Garcia Lobo, J. M., 2007. Characterization of the urease operon of *Brucella abortus* and assessment of its role in virulence of the bacterium. Infect Immun 75, 774–780.

Table 1.1. Virulence determinants of *Brucella* and their functions.

Virulence factor	Gene	Function	Reference
Alkyl hydroperoxide reductase	*ahpC* and *ahpD*	Scavenges endogenous H_2O_2 that is generated by aerobic metabolism	[79]
Base excision repair	*xthA*	Protect *Brucella* from ROS toxicity in vitro	[81]
Brucella virulence factor A	*bvfA*	Induced by phagosomal acidification and coregulated with *virB* operon suggesting a potential role in establishment of a replicative niche for *Brucella*	[90]
Catalase	*katA*	Protects from oxidative intermediates including H_2O_2 and superoxide	[78]
Cu-Zn Superoxide dismutase	*sodC*	Protects from oxidative burst of host macrophages	[77]
Cyclic β-1,2-glucans	*cgs* - Cyclic β-1,2-glucan synthetase	Mediate *Brucella* entry through lipid-rafts and controls cellular trafficking by preventing the fusion of BCVs to lysosomes	[91]
Cytochrome oxidase	*bd* ubiquinol oxidases *cbb3*-type cytochrome c oxidases	Prevention of ROS toxicity	[80]

Lipopolysaccharide	Several genes are involved in smooth LPS biosynthesis	Protects against cationic peptides, respiratory burst of macrophages, and complement activity. Prevents the induction of pro-inflammatory cytokines and impairs host adaptive immune responses against *Brucella*.	[7]
Nitric oxide reductase	*norD*	Resistance to nitrosative damage	[82]
Type IV secretion system	*virB*	Effector translocation into host cells	[58,60,63]
Urease	*ure*	Catalyzes the hydrolysis of urea to ammonia facilitating *Brucella* survival in acidic conditions	[92]

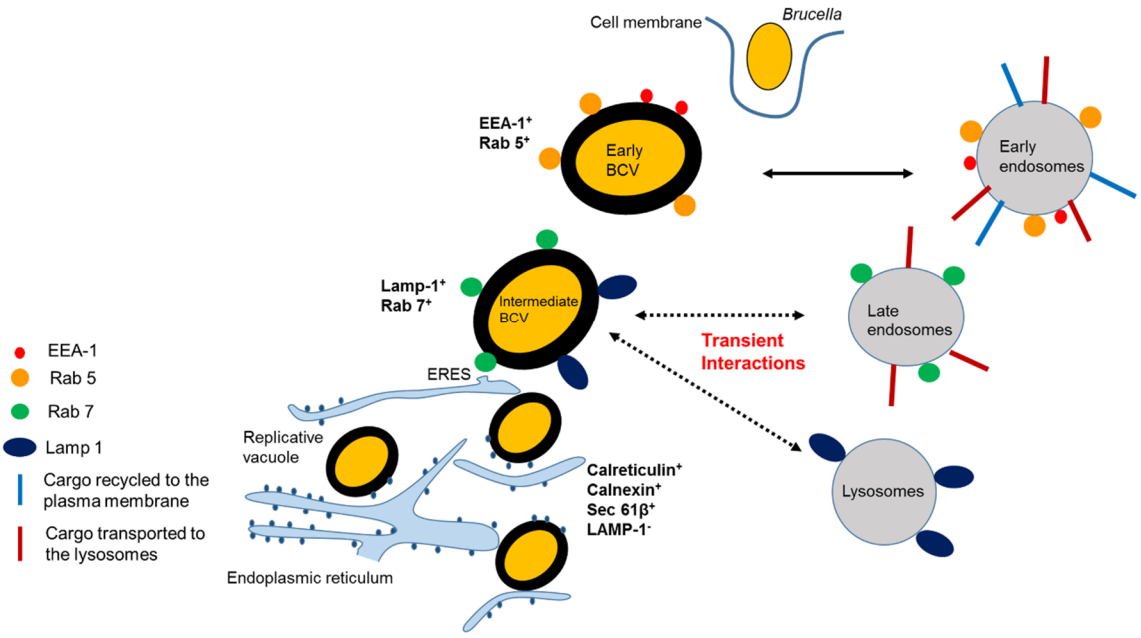

Figure 1.1. A revised model of *Brucella* intracellular trafficking. Following entry, *Brucella* is found within BCVs. These BCVs traffic along the endocytic pathway, sequentially interacting with early and late endosomes and lysosomes. Early BCVs interact with early endosomes and transiently acquire EEA-1 and Rab5. They mature into intermediate BCVs that transiently acquire LAMP-1 and Rab 7 and interact with ERES. Such interactions are necessary for the formation of an ER-derived replicative organelle. Replicative BCVs exclude LAMP-1 and acquire ER markers such as calreticulin, calnexin and sec61β. BCVs: *Brucella*-containing vacuoles; EEA-1: early endosomal antigen-1; Lamp-1: lysosomal-associated membrane protein; ERES: endoplasmic reticulum exit sites.

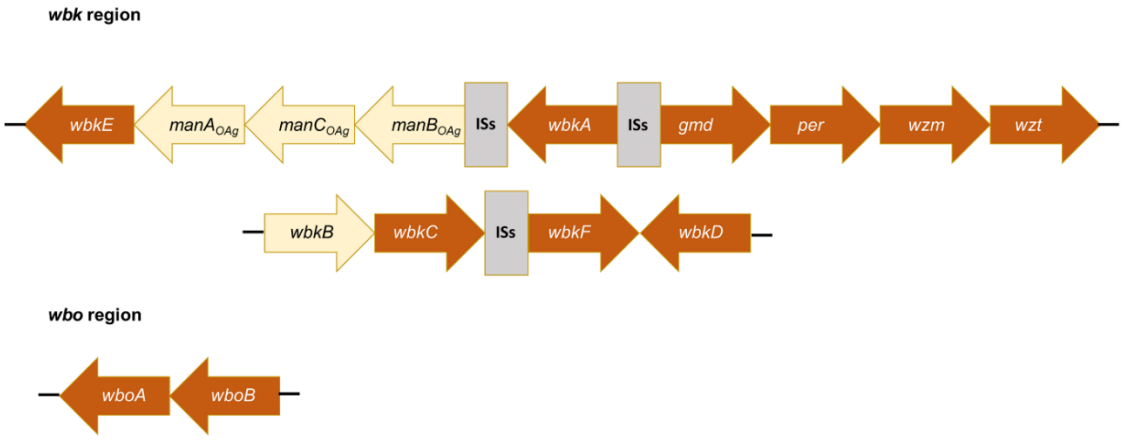

Figure 1.2. Genetic organization of the O-polysaccharide biosynthesis genes. The genes required for O-polysaccharide synthesis are located on two loci, *wbk* and *wbo*. *wbk* region contains genes coding for enzymes necessary for N-formylperosamine synthesis (*gmd*, *per*, *wbkC*), O-PS glycosyltransferases (*wbkE*, *wbkA*), the ABC transporters (*wzm*,*wzt*), the enzymes that lead to bactoprenol priming (*wbkD*, *wbkF*), as well as groups of insertion sequences (ISs). *wbo* region contains genes coding for two O-PS glycosyltransferases (*wboA* and *wboB*). Mutations in genes marked in dark brown generate rough mutants.

CHAPTER 2. DEVELOPING A VACCINE FOR HUMAN BRUCELLOSIS: WHY AREN'T WE THERE YET?

Abstract

Brucella species are facultative intracellular coccobacilli that cause one of the most frequently encountered zoonotic diseases worldwide. Most of the naturally occurring human infections are mainly caused by *B. melitensis*, *B. abortus* and *B. suis*. These species are classified as Center for Disease Control category B select agents. Brucellosis is endemic in the Mediterranean basin, the Middle East, Central Asia, Central America and parts of South America. If untreated, the infection can progress to a chronic phase that can last from a few weeks to several years. Rare complications like endocarditis, meningitis and spondylitis or death can occur. While treatment with a combination of antibiotics is usually effective, relapses are fairly common. There is no licensed vaccine available for use in humans. Safety concerns preclude the use of current live attenuated animal vaccines for human vaccination. This review focuses on the need for a human brucellosis vaccine and discusses strategies for the development of a safe and effective vaccine against human brucellosis.

Introduction

Brucellosis is a chronic infectious disease that affects several mammals, including humans. The first evidence of brucellosis in humans dates back to AD 79 as determined by bone lesions characteristic of brucellosis found in human skeletal remains in Herculaneum [1].

Cocco-bacillary forms, morphologically similar to the causative agent *Brucella*, were found in the remnants of buried carbonized cheese [2]. However, it was not until 1886 that *Brucella* was first isolated by Sir David Bruce from the spleen of a British soldier who had died of a febrile illness (Malta fever) on the Mediterranean island of Malta, not far away from Herculaneum [3]. The Roman name of Malta was 'melita' and hence the isolated organism was initially named *Micrococcus melitensis* (now *Brucella melitensis*). Later on in 1897, Bang's bacillus (now *Brucella abortus*), the causative agent of cattle brucellosis, was discovered by L.F Benhard Bang. The close homology of these two species was demonstrated by Alice Evans of the Hygiene Laboratory of the U.S. Public Health Service (now National Institutes of Health) in 1918 and the two species were regrouped under the genus *Brucella* in honor of David Bruce [4,5]. Since then, several *Brucella* species have been isolated from different mammalian hosts.

Brucella species are small, non-motile, non-spore forming, aerobic, facultative intracellular Gram-negative coccobacilli. The species classification is mainly based on their host preferences: *B. abortus* (found in cattle and buffalo), *B. melitensis* (sheep and goats) *B. suis* (pigs), *B. canis* (dogs), *B. ovis* (rams), *B. neotomae* (desert wood rats), *B. cetaceae* (whales, dolphins and porpoises), *B. pinnipedialis* (seals and walruses), and *B. microti* (voles) [6,7]. More recently, *Brucella* was isolated from a human breast implant (*B. inopinata*) [8], Australian rodents [9] and from a baboon colony (*B. papionis*) [10]. In infected animals, *Brucella* primarily targets the tissues of the reproductive tract leading to contagious abortions and infertility. Most of the naturally occuring human infections are caused by *B. melitensis*, *B. suis*, *B. abortus*, and *B. canis* in decreasing order of virulence [11]. Marine mammal *Brucella* spp. have also been isolated from humans; however, their infectivity and degree of virulence for humans is still largely unknown [8,10,12].

Need for a human brucellosis vaccine

Brucellosis is one of the most frequently encountered zoonosis [13]. Humans get infected from consumption of contaminated animal products or from direct exposure to contaminated aborted tissues. Aborted fetal tissues can contain as many as 10^{10} organisms

per cm^3 of the tissue [14]. Many cases of human infection from unintentional, accidental exposure to animal vaccines have also been reported [15]. At the site of entry, the bacteria are ingested by resident phagocytes and carried to regional lymph nodes where they multiply leading to bacteremia. *Brucella* primarily colonizes the monocytes and the macrophages of the reticulo-endothelial system (RES) including spleen, liver and lymph nodes and the reproductive system [11]. Human brucellosis is frequently misdiagnosed and underreported due to the existence of non-specific flu-like symptoms. It initially presents as a febrile illness with symptoms including undulant fever, general malaise, anorexia, arthralgia, fatigue and headache [11]. Enteric symptoms like diarrhea, abdominal pain and ulceration of Peyer's patches have been reported in cases associated with ingestion of *Brucella* [16,17]. Complications like spondylitis, arthritis, meningitis and encephalitis can ensue depending on the internal organ involved [11]. Infective endocarditis is a rare complication (1-2% of the cases) [18] that accounts for almost 80% of the deaths due to brucellosis [19,20].

Brucellosis has been eradicated from the livestock population in many parts of the world including the United States of America, Canada, Japan and Scandinavian and several other countries in Europe [13,20]. However, *Brucella* in wildlife reservoirs poses a constant threat to reemergence of the disease in food animals. As an example, *B. abortus* is prevalent in wildlife reservoirs including bison and elk populations in the Greater Yellowstone Area; almost 50% of the 4000 bison and high numbers of elks have been exposed or are infected with *B. abortus* [15]. Brucellosis is highly prevalent in the Mediterranean basin, the Middle East, Central Asia, Northern Africa, and Central and Southern parts of America [13,20]. Humans are dead-end, incidental hosts of *Brucella* and the disease incidence in humans is strongly dependent on the prevalence of brucellosis in animals. As animals are the only source of *Brucella* infection to humans, successful strategies to control the disease in animals would eliminate the risk of infection to humans. In fact, the control of brucellosis in domestic livestock by rigorous surveillance, vaccination and slaughter programs has resulted in a paralleled decrease in human brucellosis cases [21,22]. However, the developing nations still suffer under a huge disease burden of animal and human brucellosis [23]. Eradication of *Brucella* in these countries is a formidable task. Strategies used for

eradication of the disease in developed countries may not be implementable in these nations for several reasons including differences in livestock production systems, social culture, and the lack of veterinary infrastructure and financial resources. Currently, there is no safe and effective vaccine against *Brucella* infection in humans. Successful treatment of brucellosis relies on antibiotic regimens administered for a prolonged period of time. However, previous studies have shown that, despite successful therapeutic interventions, a significant number of clinically asymptomatic patients could still remain infected [24]. As *Brucella* resides in intracellular niches, relatively secluded from antibiotics, it is not surprising that relapses following antibiotic therapy are common [25,26].

A vaccine would be invaluable for controlling human disease in *Brucella*-endemic regions and for protection of people at occupational hazard including animal handlers, farmers, veterinarians, abattoir workers and lab personnel. Brucellosis is also one of the most frequently reported laboratory-acquired infections [27]. An infectious dose of 10-100 organisms has been found to be sufficient to establish disease in hosts by the aerosol route [14]. Due to their highly infectious nature and the ease of aerosol spread, *B. melitensis*, *B. suis* and *B. abortus* are considered as potential bioweapons by the CDC and listed as Class B select agents [28]. Also, *Brucella* is included in the National Institute of Allergy and Infectious Disease's Strategic Plan for Biodefense Research [29]. In fact, *B. suis* was the first organism that was weaponized in 1954 by the United States Government in an attempt to develop its offensive biowarfare. However, the United States officially terminated the development of bioweapons in 1969. As of now, there is a renewed interest in developing a vaccine as a means of direct intervention to protect the military troops and the public in the event of bioterrorism. Human brucellosis is rarely fatal; however, it is a notoriously debilitating disease that incapacitates a person [11] and causes substantial financial losses in terms of medical care for infected people [30]. A previous study has assessed that $477.7 million dollars would be incurred in the event of an aerosol exposure of 100,000 people to a bioterrorist attack [30]. This study provides economic justification for investing in preparedness measures, including vaccine development, to combat any deliberate misuse of *Brucella* as a bioweapon [30].

Currently used animal vaccines

Currently, three live vaccines, *B. abortus* strains 19 and RB51 for cattle and *B. melitensis* Rev 1 for sheep and goats, are used to control brucellosis [31,32]. However, strain 19 and Rev 1 retain some pathogenicity while RB51 is only 70% effective in cattle and its efficacy in pigs, sheep and goats is questionable [33-36]. None of the vaccines induce a lifelong 100% protection against the infection that is encountered in heavily infected herds/flocks.

B. abortus Strain 19 (S19)

This strain was isolated from milk of a Jersey cow in the early twentieth century. It is an attenuated strain obtained spontaneously after the virulent *B. abortus* was maintained at room temperature for one year [37]. It exhibits a phenotypic sensitivity to erythritol owing to the deletion of a region of 702 bp encompassing erythritol catabolic genes [38]. This strain has been used worldwide and has been instrumental in achieving a significant reduction in the prevalence of brucellosis in cattle. The use of S19 was discontinued in United States after the introduction of the rough strain RB51 in the mid-1990s. This vaccine is still being used in several countries [39, 40]. One drawback of this vaccine is the induction of anti-O-polysaccharide (O-PS) antibodies in the vaccinated animals. These antibodies interfere in the diagnostic assays that are routinely used to detect infected animals. The persistence of these antibodies is dependent on the age of the vaccinated animal, the dose and the route of immunization. Vaccination of adult cattle with a standard dose of 3×10^{10} CFU-equivalent of S19 induces antibodies that persist for upto 10-11 months post-vaccination [41,42]. Calfhood vaccination shortens the duration of antibody persistence; however, a lower level of protection is induced in calves than in adults [31]. This strain is also associated with low abortifacient (approximately 5%) potential when used for vaccinating pregnant animals [43,44]. When used in adults, it results in udder infections in 2% of the animals which also shed the bacteria in milk [45,46]. Less frequently, S19 vaccination results in the development of arthropathy [47]. S19 vaccine is

virulent for humans; several cases of human disease have been due to accidental inoculation of S19 vaccine [32,33,48].

B. melitensis Rev 1

B melitensis Rev 1 is a live attenuated strain developed in 1957 that has been successful in preventing brucellosis in small ruminants [49]. It protects against both *B. melitensis* infection in sheep and goats and *B. ovis* infection in rams [49,50]. Controlled studies show that Rev 1 vaccination can induce high levels of immunity that can last longer than 4 years in goats and for at least 2.5 years in sheep [49]. In spite of being attenuated when compared with field strains, Rev 1 retains some virulence [51]. It is abortifacient and the abortion rate can be as high as 80% if the animals are vaccinated in their second to third month of pregnancy [49]. To avoid abortions, animals are vaccinated prior to the first gestation at 3-7 months of age. Also, conjunctival administration can substantially reduce the abortion rate [52]. Rev 1 vaccine has been found to be of very low virulence in rams [53]. Being a smooth strain, vaccination with Rev 1 results in induction of antibodies against the O-PS that interfere with the serodiagnosis. Also, although rarely excreted in sheep milk, Rev 1 can be secreted in goat milk for several weeks to months posing a hazard for the farmers [52]. Despite being attenuated in animals, Rev 1 is capable of infecting humans [54].

B. abortus RB51

B. abortus strain RB51 was licensed for use in cattle by Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture (USDA), in February 1996 [55]. This vaccine is being currently used in a number of countries including the United States. It induces a good level of protection in cattle; however, its protective efficacy in swine, sheep and goats is still unclear [33-36]. *B. abortus* RB51 is a genetically stable, live, attenuated, spontaneous mutant of *B. abortus* 2308 obtained after repeated *in vitro* passages in the presence of penicillin and rifampin [56]. The *wboA* gene, that encodes a glycosyltransferase required for O-PS synthesis in *Brucella*, is disrupted by an *IS711*

element in RB51 [57]. Although RB51 synthesizes low levels of M-like O-chain [58], it does not induce any measurable amounts of anti-O-PS antibodies that might interfere in smooth-LPS serological tests [59]. Also, it possesses low virulence and does not induce abortions in the vaccinated animals [60]. RB51 affords a similar level of protection as S19 in cattle [61]; however, it is considered less virulent than S19 on the basis of results from animal testing. Calves are generally vaccinated subcutaneously (SC) at 4-12 months of age with a dose of $1\text{-}3.4\times10^{10}$ organisms without adverse effects. A vaccine dose of 10^9 organisms can be safely used to vaccinate pregnant cattle via SC route without the induction of abortions or placentitis [62]. Despite its efficacy in cattle, two major drawbacks preclude its use for vaccination of humans. Firstly, accidental exposure of humans to RB51 is known to cause local and systemic adverse reactions [55,63]; secondly, RB51 is resistant to rifampin which is an effective antibiotic for treating brucellosis [56]. Although RB51 is genetically stable, the exact nature of mutations affecting its attenuation characteristic is not known. Previous studies document that complementing strain RB51 with a functional *wboA* gene does not restore the smooth phenotype suggesting that mutations in several genes might be contributing to its rough colony morphology and attenuation [64].

History of vaccination of humans against brucellosis

Previously, numerous attempts have been made to vaccinate humans against brucellosis. Formerly, a strain derived from Strain 19, Strain 19-BA, was used extensively during a period extending from 1940-1960 to immunize at least 3 million people in the former USSR [65]. People were vaccinated with 1×10^9 cells via skin scarification. It delivered partial protection that did not result in long-lasting immunity and frequent re-immunizations were required. Local adverse reactions including hyperaemia and induration were exhibited by 76% of the vaccinates while generalized symptoms like headache, lethargy and fever occurred in 3 to 7% of the immunized people [65,66]. Also, vaccination with 19-BA was associated with the appearance of variable degrees of hypersensitivity reactions especially with repeated vaccinations [65,66]. A decline of 60%

in acute human brucellosis cases during 1952-1958 was attributed to the use of 19-BA vaccine [65,66].

Other live attenuated strains like *B. abortus* VA19 and *B. abortus* 104M were used in the former USSR and China, respectively [67]. They were administered intradermally or in aerosolized forms [67]. Severe adverse reactions were associated with their improper use or use in sensitive individuals [67]. On account of safety issues, emphasis was shifted towards non-living vaccines. Vaccines based on subcellular fractions including a peptidoglycan fraction (PI) and an extracted protein-polysaccharide fraction (BCV-*Brucella* chemical vaccine) were used in France and Russia, respectively [68]. PI was a phenol-insoluble residue obtained from lipid-extracted *B. melitensis* M15 [69,70,71]. Subsequently, *B. abortus* S19 was used for developing PI. Two doses of PI at 2 week interval were administered SC to high-risk populations including laboratory workers [69]. The vaccine was non-toxic and less reactogenic. Protection in vaccinates lasted for about 18-24 months [69,70]. Although PI was used as a vaccine against brucellosis for almost two decades, conclusive evidence of its efficacy from controlled clinical trials does not exist [72]. This vaccine is no longer in production.

Similarly, an acetic acid extracted protein-polysaccharide fraction of cell wall was developed in USSR [73,74]. It was prepared from *B. abortus* strain 19-BA and was administered via intra-muscular (IM) route in humans [73,74]. Studies involving the use of 75,000 vaccine doses in Kazakhstan indicated that the protective efficacy of BCV was similar to that of strain 19-BA [73,75]. However, BCV was shown to have lower reactogenicity than strain 19-BA [73,75]. The efficacy of this vaccine has not been rigorously tested under controlled clinical trials [75].

Immune responses against *Brucella*

Immune response studies and gene expression patterns in mouse and macrophage cell lines during *Brucella* infection have contributed significantly to our current knowledge about immunity to *Brucella*. Both humoral [76-80] and cell-mediated responses [80,81] participate in immunity against brucellosis. However, cell-mediated immunity (CMI) plays

a major role in acquired resistance against *Brucella* infection [81]. Th1 type of immune response is considered essential for clearing *Brucella* from mice [81-83]. Interferon- γ (IFN-γ) is an important cytokine for resistance to *Brucella* infection. It is secreted by T cells and natural killer (NK) cells and activates the bactericidal function of macrophages by promoting the production of reactive oxygen intermediates, enhancing the production of cytokines, and by inducing isotype switching to immunoglobulin (Ig)G2a and IgG3 [81,84]. Consequently, IFN-γ-knockout mice or mice lacking interferon regulatory factor -1 (IRF-1) are unable to control *Brucella* and succumb to infection within 6 weeks [84,85]. Tumor necrosis factor-α (TNF-α) also plays a similar role of enhancing the bactericidal activity of macrophages [86]. Previous studies document the role of interleukin-12 (IL-12) in resistance against brucellosis; in a mouse model, depletion of IL-12 before *Brucella* infection exacerbated the infection and enhanced the chronicity of the disease [87].

Both $CD4^+$ and $CD8^+$ T lymphocytes help to control *Brucella* infection as shown by specific T cell depletion experiments using T cell-specific monoclonal antibodies and also by using gene knock-out mice [80,81]. Importantly, both cell populations are sources of IFN-γ which helps to control the intracellular growth of *Brucella* [81]. Studies in human primary cells have helped to identify some aspects of immune responses that are restricted to humans. Gamma delta (γδ) T cells appear to play a role in brucellosis that is especially prominent in humans. A human-specific population of γδ T cells (V γ9δ2) can kill *B. suis*-infected cells by releasing microbicidal peptides [88]. γδ T cells might also exert their protective effect in humans by release of IFN-γ and through cytotoxic activity [89]. Also, previous studies showed that human natural killer (NK) cells can mediate killing of *B. suis*-infected cells in a contact-dependent manner [90]. While human NK cells play a role against *Brucella* infection, murine NK cells are not important in controlling brucellosis [91].

B. abortus infection also induces the production of IL-10 which inhibits the microbicidal function of macrophages and antagonizes the activity of IFN-γ [92,93]. $CD4^+$ T cells have been identified as the major source of IL-10 during the early phase of *Brucella* infection. A recent study demonstrates that mice lacking IL-10 are better able to control *Brucella* infection. Also, blocking IL-10 production restricts the ability of *Brucella* to escape

$LAMP1^+$ late endosomes and replicate intracellularly thereby limiting the establishment of chronic infection [94].

The significance of humoral immunity has been repeatedly demonstrated, both in murine model of brucellosis as well as in natural hosts [76-80]. IgG2a and IgG3 are the dominant antibody isotypes detected in infected mice and natural hosts, again emphasizing the prominent role of Th1 immunity against brucellosis [95]. Passive transfer of anti-LPS sera as well as *B. abortus* O-PS specific monoclonal antibodies protect mice against challenge with virulent *Brucella* species [76, 78-80]. These antibodies presumably play a direct role in opsonization; antibody-mediated opsonization enhances the phagocytic uptake and intracellular killing of *Brucella* [96].

Strategies for optimizing the design of a brucellosis vaccine for use in humans

A major challenge for development of a vaccine against human brucellosis is the absence of well-established correlates of protection. However, extensive studies with mouse models have helped to identify definite indices of immune responses that are required for protection against *Brucella*. An ideal brucellosis vaccine should be avirulent and effective, and should be able to provide long-term protection, preferably with a single dose.

Vaccine safety and protective response

Historically, live attenuated vaccine candidates, including the vaccines currently in animal use, have delivered the most promising results against *Brucella* infection [97]. Live attenuated organisms mimic the infection caused by live pathogens and can deliver multiple antigens for processing and presentation by dendritic cells (DCs) and macrophages for stimulation of a robust immunity. However, lack of knowledge about the molecular basis of attenuation as well as safety risks preclude the use of currently used field vaccines in humans. Recent years have seen a considerable amount of research on the identification of genes required for *Brucella* to successfully survive in the host and cause

disease [98-100]. This knowledge can be exploited to design a safe and effective vaccine by making the organism innocuous while still able to traffic within host cells and interact with host components for induction of optimum protection. Inactivation of genes involved in virulence and survival have yielded several promising *Brucella* attenuated strains [101-104]. However, it is crucial that prospective vaccine candidates should not possess any residual virulence. Such a vaccine design is often problematic as genetic mutations that severely affect virulence may attenuate the organisms to levels where they are unable to induce sufficient protection. Following a careful assessment of the residual virulence, the attenuated candidates are evaluated for vaccine efficacy using laboratory animal models. Previous studies have indicated that some level of persistence is essential for a vaccine to stimulate an adaptive immune response [105-108]. Attenuated strains that undergo rapid clearance are unable to produce sufficient protection. However, attenuated strains that exhibit incomplete clearance from the host may be of questionable safety and might induce symptoms of full-blown brucellosis. Only the vaccine candidates that meet the desired level of attenuation and immunogenicity in mice can be evaluated rigorously for safety and protective efficacy in non-human primates. It is also important to take into account the genetic predispositions to potential side effects or that can influence vaccine immunogenicity.

In the absence of identification of genes that can enhance persistence without compromising safety, several alternate strategies such as encapsulation can be employed. Encapsulation of highly attenuated organisms that are otherwise rapidly cleared can enhance the immune responses and protection against pathogens by providing gradual timed release of the vaccine (discussed later in section ‘Mucosal delivery of vaccines’).

Alternatively, attenuated strains that exhibit optimal persistence can be further subjected to gamma-irradiation to enhance their safety by rendering them incapable of reverting to virulence [109-113]. An optimal dose of gamma-rays can abolish the replicative ability of organisms by creating sufficient number of breaks in the genomic DNA. Gamma-ray attenuation of microbes leaves a large portion of the genomic nucleic acid intact for transcriptional and translational activity. This ensures the downstream effectiveness of the elicited immune responses against the expressed proteins of the pathogen. Several previous

studies document that gamma-irradiated *Brucella* strains can induce potent protection [109, 111-113]. Gamma-irradiated strains are metabolically active; microbial metabolism is a potential viability-associated PAMP (vita-PAMP) that helps the host system to differentiate between live and killed microbes [114]. vita-PAMPs signal microbial viability that is identified by the innate immune system and consequently, the host system can perceive the level of threat and launch a proportionally potent adaptive immune response against the invading pathogen [114]. Killed vaccines lack vita-PAMPs and are only weakly immunogenic [115]. Hence, while being safe, gamma-ray-attenuated vaccines possess comparable efficacy to live vaccines.

Another concept that assures a safe vaccine design is the 'killed-but-metabolically-active' (KBMA) approach. This approach has yielded several safe, potential vaccines for pathogens such as *Listeria monocytogenes*, *Salmonella* Typhimurium, *Leishmania infantum*, and *Bacillus anthracis* [116-119]. According to the KBMA concept, bacterial strains are mutated in the nucleotide repair system to make them exceedingly sensitive to DNA damage induced via ultraviolet (UV) light [116]. Consequently, these mutants can be inactivated using a synthetic psoralen that causes pyrimidine residues to cross-link on exposure to UV radiation. Organisms are unable to repair this psoralen-induced DNA damage as they are mutated in the ABC excision endonuclease (excinuclease) complex that mediates nucleotide excision repair [120]. These vaccines are safe and induce protection in the vaccinated hosts [116-119].

With increasing awareness of vaccine-associated potential adverse effects, rigorous controlled studies need to be undertaken to gather convincing evidence of vaccine safety and enable vaccine acceptance. With this in mind, significant efforts have been focused on the development of subunit and DNA vaccines. These vaccines are well-defined, nonviable and unable to cause disease. However, subunit vaccines exhibit weak immunogenicity mostly as a result of the difficulty to target them to appropriate tissues or to mimic the intracellular trafficking pattern of live infectious pathogen and stimulate various facets of the host defense system. The immunogenicity of the subunit vaccines can be potentiated by inclusion of supplements that can enhance the overall vaccine efficacy. However, even with the use of such immunopotentiators (adjuvants), the induced CMI response may not

be robust enough to provide protection against *Brucella* infection (CpG adjuvants are discussed in the next page).

Alternatively, DNA vaccination can generate both humoral and CMI in the vaccinated subjects. DNA vaccines can induce major histocompatibility complex (MHC) class I-restricted responses and mimic the effects of live attenuated vaccines [121]. This feature is advantageous when compared to conventional protein-based vaccines. Also, DNA vaccines do not possess the inherent risk of potential bacterial reversion as seen with live attenuated vaccines. DNA vaccines are biologically stable, their manufacturing is cost effective and simple and they do not require cold-chain maintenance as they can be transported in lyophilized form [121]. Previous studies have shown that DNA vaccines encoding protective antigens such as lumazine synthase (BLS), outer membrane protein (Omp) 31 and Cu, Zn superoxide dismutase (SOD) of *Brucella* provide protection against virulent *Brucella* challenge in mice [122-124]; the level of protection induced is similar to live attenuated reference vaccines used in these studies. This protection is attributed to the production of antigen-specific humoral and CMI responses, primarily of Th1 type. DNA vaccine design also allows for the inclusion of multiple protective antigens. Immunization with a divalent vaccine encoding L7/L12 and Omp 16 or a trivalent vaccine encoding L7/L12, BCSP31 and SOD confers protection at comparable levels to that induced by licensed field vaccines [125,126].

One of the principal advantages of DNA vaccines is that they be easily tailored to induce desired immune responses. Several complementary strategies to alter the qualitative and quantitative aspects of immune responses and enhance the potency of DNA vaccines have been developed. Genes encoding cytokines, chemokines and co-stimulatory molecules can be incorporated in DNA vaccines to amplify the induced immune responses as well as control the type of immunity. As an example, while a range of T-cell responses including Th1, Th2 and cytotoxic T lymphocytes (CTLs) are induced using granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-12 primarily induces CTL responses [127]. Also, a combination of cytokines can be administered for the synergistic stimulation of T-cell polarized immunity. Previous studies document that a combination of GM-CSF, TNF-α and IL-12 enhances the protective efficacy of a vaccine [128,129]. GM-CSF increases the

number as well as function of DCs, while IL-12 and TNF-α upregulate the expression of IL-12 receptor and IFN-γ [129]. Also, genes for co-stimulatory molecules can be incorporated for maximizing the vaccine efficacy. A combination of IL-12 and B7-1 acts synergistically and enhances both the antigen-specific T helper cell proliferation and CTL responses [130,131]. Thus, cytokines and costimulatory DNA that can induce Th1-biased immune responses can be exploited for potentially enhancing the vaccine efficacy against brucellosis.

Alternatively, adjuvants can be used to induce relevant cytokines and to upregulate the expression of co-stimulatory molecules leading to increased vaccine efficacy. In particular, CpG oligodeoxynucleotide (ODN) is considered as one of the most promising adjuvant for the development of improved vaccines against infectious diseases including brucellosis. It can be used as an adjuvant in both antigen-based conventional vaccines as well as DNA vaccines against brucellosis. ODNs are immunostimulatory DNA sequences containing unmethylated CpG dinucleotide motifs that can directly activate human B-cells to proliferate and differentiate into IgG producing B-cells [132]. They exert their effect as a polyclonal activator via TLR9. Overall, CpG ODNs induce a cytokine profile that is characteristic of Th1 type of immune response including higher production of IL-12 and IFN-γ [133]. They enhance $CD4^+$ T cells, $CD8^+$ T cells and antibody responses to a variety to pathogens including *Brucella* [134]. All the published protection studies with subunit and DNA vaccines were conducted in mouse models; whether these vaccines perform similarly in target host species remains to be tested.

Before any of these strategies can be effectively translated into applicable vaccine technology, associated potential risks and long term ethical implications should be carefully considered. As an example, one major drawback of using cytokine-encoding plasmids is that they can persist in the host for months or years. The overproduction of one type of effector cytokine can skew the balance of Th1 and Th2 immune responses, disrupt the immune homeostasis and alter the host's susceptibility to various infections. As multicomponent vaccines become more common, the possibility for detrimental adverse effects will also increase. Rigorous preclinical studies are needed to adequately establish the safety of any future vaccine candidate.

Induction of immunity at mucosal surfaces

Most infectious agents infect humans via mucosal sites, principally the digestive and respiratory tracts. Therefore, mucosal immunity constitutes a very critical part of overall protective responses against the invading microbes. Vaccines that elicit robust immunity at mucosal sites would prevent colonization of mucosal surfaces and enhance resistance by limiting the entry of pathogens beyond the mucosa. However, most licensed vaccines are administered parenterally and target the systemic immune system. They either elicit only a weak mucosal response or altogether fail to induce protective mucosal immunity [135]. The understanding that mucosal vaccination is required to fortify defenses at mucosal sites and protect against infections has renewed interest in the development of efficacious mucosal vaccines. Mucosal immunization can induce the expression of mucosal and systemic homing receptors in lymphocytes and can prime the host immune system for subsequent mucosal as well as systemic recall responses leading to enhanced protection at both sites. However, direct mucosal immunization is challenging; the vaccine dose that actually enters the body cannot be accurately assessed due to the difficulty of capturing and analyzing the functionality of mucosal antibodies as well as mucosal T cells [136]. Also, mucosal vaccines have to face a similar range of host innate defenses as mucosal pathogens do such as the degradative lytic enzymes, epithelial barriers and mucus secretions. Vaccines delivered via mucosal routes tend to become diluted in mucosal secretions limiting effective deposition onto mucosal epithelium [136]. Also, they can become stuck in mucus and subsequently degraded by proteases [136]. To overcome these limitations, relatively large doses are required to stimulate immune responses when compared to parenteral vaccination strategy [136].

Despite the complications associated with mucosal vaccination, there are some mucosal vaccines that are approved for human use. Among those are the live attenuated poliovirus and *Salmonella typhi* oral vaccines that are both derived from pathogens that exploit M cell transport to invade the enteric lymphoid tissues [137-140]. The effectiveness of these live vaccines is partly a result of their ability to mimic the mucosal invaders in adhering to

mucosal surfaces, invading the organized mucosal lymphoid tissues and stimulating innate and adaptive immune responses against the target pathogen [136].

Prevention of mucosal transmission should be a goal in developing an effective brucellosis vaccine as *Brucella* is mainly transmitted by mucosal routes. However, as human brucellosis is a systemic disease, an ideal vaccine should be able to induce both mucosal as well as systemic protection. The choice of vaccination route is partly dependent on the expected site of challenge. Human brucellosis is often acquired through aerosol route; protection against inhalational challenge should be considered while evaluating the vaccine efficacy of potential candidates for humans. Several *Brucella* investigators have demonstrated the generation of enhanced protection against intra-nasal (IN) challenge infection with virulent *Brucella* spp. in mice following IN and oral immunization with live attenuated *Brucella* vaccines [113,141-143]. These results are extremely promising; future studies need to focus on the strategies to overcome the physiological barriers at mucosal routes, specific targeting of antigen-presenting cells (APCs) at inductive sites for immune activation and modulating the kinetics of antigen delivery to promote long-lasting protection. One promising approach of mucosal vaccination involves entrapping antigens within synthetic polymeric particles designed to mimic immunogenic properties of natural pathogens. These carriers can protect the antigens from degradation, selectively target them to preferential sites of antigen uptake, and release the antigens slowly over a prolonged period of time (discussed in ‘mucosal delivery of vaccines’).

Mucosal delivery of vaccines

Notable advances to improve the delivery of vaccines to mucosal inductive sites have focused on the use of particulate systems. These include carrier particles like poly (lactide co-glycolide) (PLG), liposomes, chitosan, virosomes, ISCOMs etc. for controlled-release of antigens and also for eliminating the need for booster immunizations [144,145]. Particulate carriers prepared from PLG are one of the most widely used delivery platforms for vaccines. They are biodegradable, biocompatible, and have previously been used as suture materials and other controlled-release therapeutic drug delivery systems [146,147].

Particulate delivery systems potentiate vaccine efficacy through the efficient uptake of the antigen by the APCs and subsequent delivery to lymphoid tissues [148]. The encapsulated antigen can be released in a pattern to maximize the elicited immune response by varying the release kinetics of the particles. Optimum rate of antigen release would result in extended antigen presentation to the host immune system [149] potentially allowing for a single-shot vaccine formulation to induce long-lasting effective immunity.

One important determinant of efficient antigen uptake and its cellular trafficking as well as immune stimulation is the size of the encapsulating particles. Particles of 20-200 nm induce a Th1-biased immune response following receptor-mediated endocytosis while particles >0.5 µm stimulate humoral immunity following uptake by phagocytosis or pinocytosis [150]. Simultaneous administration of particles of distinct size distributions can be exploited to release the antigen in a pulsed pattern analogous to primary and booster immunizations. Following oral immunization, particles of 5 µm-10 µm are taken up by Peyer's patches and remain detectable for about 35 days [151]. In contrast, particles of <5 µm are found in draining lymph nodes and spleen [151]. Also, the surfaces of particles can be tailored to enhance particle diffusion through the mucosa as well as transcytosis by M cells [152]. Following mucosal entry, the infectious agents are delivered through the intestinal Peyer's Patch Follicle-Associated Epithelium (FAE) and Nasal Associated Lymphoid Tissue (NALT) by the activity of M cells [153]. For efficient uptake, vaccine particles should mimic M cell-invasive pathogens. Particles that are small (<1 µm) and adherent to M cells are taken up efficiently and transported to Peyer's patches. Ligands can be exploited for targeting particles to M cells. However, only a limited number of receptors and their ligands have been identified; most of them including pathogen recognition receptors (PRRs) are expressed by both M cells as well as by enterocytes. Previous studies have demonstrated that *Ulex europaeus* agglutinin 1 (UEA-1), a lectin specific for α-L-fucose residues, selectively binds to the apical surface of murine M cells [153]. Coating particles with such ligands can increase the antigen uptake and transport to mucosal inductive sites, thus enhancing the protective efficacy of vaccines [153]. Oral vaccination with killed *Helicobacter pylori* and UEA-1 resulted in the induction of protection against challenge infection [154]. The glycosylation pattern of M cells is species-restricted; it

remains to be determined whether UEA-1 can be used to effectively target human M cells [155].

One of the main advantages of particulate delivery for mucosal immunization is the protection of the antigen from degradation by extracellular proteolytic enzymes. This would help reduce the antigen dose that is required for immune activation especially following oral immunization. Also, various immunopotentiators such as cytokines can be codelivered with encapsulated antigens to selectively modulate and enhance the potency of elicited immune responses. Many of the cytokines have a short half-life *in vivo* and cause adverse effects if administered at high doses. Sustained release of cytokines following their encapsulation serves to overcome these limitations.

Microparticle-delivery of *Brucella* holds potential to enhance the vaccine efficacy. Microencapsulation of *Brucella* antigens has been successful in inducing potent immunity in animal models [156-160]. Studies with *Brucella* have also demonstrated that microencapsulated delivery of live attenuated *Brucella* results in the induction of sustained antibody responses, a Th1-biased immunity and enhanced protection against challenge when compared to non-encapsulated counterparts [156,158,160].

Hence, microencapsulation is an attractive alternative that can optimally exploit controlled-sustained release and preferential targeting for the development of a protective human brucellosis vaccine.

Differentiating vaccinated humans from infected humans

The differentiation of infected humans from vaccinated ones can be challenging; however, it is advantageous for any future potential human vaccine to possess this capability. Diagnostic methods for brucellosis are mainly based on serology and detect antibodies to the O-PS of smooth LPS. Vaccination of hosts with rough strains prevents the development of smooth LPS-specific antibodies; however, absence of O-PS makes the rough strains more susceptible to complement-mediated lysis, limiting their persistence in the host and vaccine efficacy [97]. At least in some animal species, these anti-O-PS antibodies play a role in enhancing protection against infections with *B. abortus*, *B. melitensis* and *B. suis*

[78-80]. In many cases, *Brucella* strains affected in their ability to produce smooth LPS are unable to induce enhanced protection when compared with live smooth strains. Previous studies have shown that inducing O-PS expression in strain RB51 by complementing it with a functional *wboA* gene enhances its protective efficacy against virulent *B. abortus* and *B. melitensis* challenge [64,161]. Efforts to induce O-PS expression in rough strains that exhibit optimum persistence might yield a safe vaccine with enhanced efficacy. Also, smooth strains attenuated in virulence can be assessed for their safety and vaccine efficacy. Vaccine safety can also be enhanced by subjecting the engineered strains to gamma-rays to render them non-replicative. However, the use of O-PS expressing *Brucella* strains as vaccines will require the development of alternate diagnostic tests for distinguishing infected hosts from vaccinated ones.

Previous studies have shown that several strategies can minimize the intensity and the duration of the antibody responses against smooth LPS. These include reducing the vaccine dose, vaccination via conjunctival route and vaccinating young hosts; however, these approaches do not completely eliminate the possibility of serologic interference. Another way is to create genetic markers by either heterologous expression of GFP or by deletion of specific genes in vaccine strains to allow for the differentiation of vaccinated hosts from infected hosts. Previous studies show that expression of a heterologous gene in *Brucella* vaccine strain provides a marker to differentiate the vaccine from the wild-type *Brucella* spp. [162]. The authors modified the O-PS of *Brucella* to produce a distinct immunogenic epitope that does not react with the antibodies from naturally infected animals [162]. Such novel approaches will permit the use of smooth LPS intact vaccines for stimulating robust immune responses leading to a long-lasting protection.

Conclusions

An efficiently attenuated *Brucella* strain with improved stability and safety is required for use as a human vaccine. However, the rational design of such a vaccine is impeded by our current lack of understanding of the precise immune correlates of protection against brucellosis. As such, an effective vaccine would need to engage multiple immune

mechanisms and mimic natural pathogens, without inducing disease. Numerous strategies can be exploited to enhance the safety and immunogenicity of the vaccine, influence the quality of the elicited immune response and circumvent any inhibitory immune mechanisms. Important challenges lie ahead: we would have to rely on animal models to examine the efficacy of vaccine candidates as clinical challenge in humans would face ethical objections. In the coming years, it is likely that a combination of several of the approaches reviewed here will allow the development of a safe and effective vaccine for human brucellosis.

References

[1] Kay, G. L., Sergeant, M. J., Giuffra, V., Bandiera, P., Milanese, M., Bramanti, B., Bianucci, R., Pallen, M. J., 2014. Recovery of a medieval *Brucella melitensis* genome using shotgun metagenomics. MBio 5, e01337-14.

[2] Capasso, L., 2002. Bacteria in two-millennia-old cheese, and related epizoonoses in roman populations. J Infect 45, 122–127.

[3] Godfroid, J., Cloeckaert, A., Liautard, J. P., Kohler, S., Fretin, D., Walravens, K., Garin-Bastuji, B., Letesson, J. J., 2005. From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet Res 36, 313–326.

[4] Meyer, K., Show, E., 1920. A comparison of the morphological, cultural, and biochemical characteristics of *B. abortus* and *B. melitensis*; studies of the genus *Brucella*. J Infect Dis 27, 73–184.

[5] Nicoletti, P., 2002. A short history of brucellosis. Vet Microbiol 90, 5–9.

[6] Verger, J. M., Grimont, F., Grimont, P. A., Grayon, M., 1987. Taxonomy of the genus *Brucella*. Ann Inst Pasteur Microbiol 138, 235–238.

[7] Verger, J. M., Grayon, M., Cloeckaert, A., Lefevre, M., Ageron, E., Grimont, F., 2000. Classification of *Brucella* strains isolated from marine mammals using DNA-DNA hybridization and ribotyping. Res Microbiol 151, 797–799.

[8] Scholz, H. C., Nockler, K., Gollner, C., Bahn, P., Vergnaud, G., Tomaso, H., Al Dahouk, S., Kampfer, P., Cloeckaert, A., Maquart, M., Zygmunt, M. S., Whatmore, A. M., Pfeffer, M., Huber, B., Busse, H. J., De, B. K., 2010. *Brucella inopinata* sp. nov., isolated from a breast implant infection. Int J Syst Evol Microbiol 60, 801–8.

[9] Tiller, R. V., Gee, J. E., Frace, M. A., Taylor, T. K., Setubal, J. C., Hoffmaster, A. R., De, B. K., 2010a. Characterization of novel *Brucella* strains originating from wild native rodent species in North Queensland, Australia. Appl Environ Microbiol 76, 5837–5845.

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

[11] Young, E. J., 2000. *Brucella* species, in: Principles and Practice of Infectious Disease. Mandel, G. L., Bennett, J. E., and Dolin, R. (Eds.), Churchill-Livingstone, Philadelphia, 2386–2393.

[12] Whatmore, A. M., Dawson, C. E., Groussaud, P., Koylass, M. S., King, A. C., Shankster, S. J., 2008. Marine mammal *Brucella* genotype associated with zoonotic infection. Emerg Infect Dis 14, 517–18.

[13] Pappas, G., Panagopoulou, P., Akritidis, N., Christou, L., Tsianos, E. V., 2006b. The new global map of human brucellosis. Lancet Infect Dis 6, 91-99.

[14] Alexander, B., Schnurrenberger, P. R., Brown, R. R., 1981. Numbers of *Brucella abortus* in the placenta, umbilicus and fetal fluid of two naturally infected cows. Vet Rec 108, 500.

[15] Acha, P. N., Szyfres, B., 1980. Zoonosis and communicable diseases common to man and animals. Pan American Health Organization, Washington DC, 28-35.

[16] Petrella, R., Young, E. J., 1988. Acute *Brucella* ileitis. Am J Gastroenterol 83, 80-82.

[17] Thapar, M. K., Young, E. J., 1986. Urban outbreak of goat cheese brucellosis. Pediatr Inf Dis 5, 640-643.

[18] Ferreira, P., Gama, P., Correia, J., Nunes, L., Pipa, J., Nascimento, C., Alexandre, J. C., Henriques, P., Santos, J. O., 2008. *Brucella* endocarditis-case report and literature review. Rev Port Cardiol 27, 1309-15.

[19] Peery, T. M., Belter, L. F., 1960. Brucellosis and heart disease. II Fatal brucellosis: A review of literature and report of new cases. Am J Pathol 36, 673-697.

[20] Pappas, G., Akritidis, N., Bosilkovski, M., Tsianos, E., 2005a. Brucellosis. New Eng J Med 352, 2325-2336.

[21] Taleski, V., Zerva, L., Kantardjiev, T., Cvetnic, Z., Erski-Biljic, M., Nikolovski, B., Bosnjakovski, J., Katalinic-Jankovic, V., Panteliadou, A., Stojkoski, S., Kirandziski, T., 2002. An overview of the epidemiology and epizootology of brucellosis in selected countries of central and southeast Europe. Vet Microbiol 90, 147-155.

[22] Minas, A., Minas, M., Stournara, A., Tselepidis, S., 2004. The effects of Rev-1 vaccination of sheep and goats on human brucellosis in Greece. Prev Vet Med 64, 41-47.

[23] Cutler, S. J., Whatmore, A. M., Commander, N. J., 2005. Brucellosis- new aspects of an old disease. J Appl Microbiol 98, 1270-1281.

[24] Navarro, E., Segura, J. C., Castano, M. J., Solera, J., 2006. Use of real-time quantitative polymerase chain reaction to monitor the evolution of *Brucella melitensis* DNA load during therapy and post-therapy follow-up in patients with brucellosis. Clin Infect Dis 42, 1266-1273.

[25] Memish, Z., Mah, M. W., Al Mahmoud, S., Al Shaalan, M., Khan, M. Y., 2000. *Brucella* bacteraemia: clinical and laboratory observations in 160 patients. J Infect 40, 59-63.

[26] Hall, W. H., 1990. Modern chemotherapy for brucellosis in humans. Rev Infect Dis 12, 1060-99.

[27] Robichaud, S., Libman, M., Behr, M., Rubin, E. E., 2004. Prevention of laboratory-acquired brucellosis. Clin Infect Dis 38, e119-e122.

[28] United States Department of Health and Human Services, 2002a, 42 CFR Part 1003 Possession, use and transfer of select agents and toxins, Fed. Reg. 240, 76886-76905.

[29] United States Department of Health and Human Services, 2002b, NIAID strategic plan for biodefense research. NIH Publication No. 03-5306.

[30] Kaufmann, A. F., Meltzer, M. I., Schmid, G. P., 1997. The economic impact of a bioterrorist attack: are prevention and postattack intervention programs justifiable? Emerg Infect Dis 3, 83-94.

[31] Nicoletti, P., 1990. Vaccination. In: Animal brucellosis. Nielsen, K., Duncan, J. R. (Eds.), CRC Press, Boca Raton, 284-299.

[32] Elberg, S. S., Faunce, W. K., 1957. Immunization against *Brucella* infection. IV. Immunity conferred on goats by a non-dependent mutant from a streptomycin dependent mutant strain of *Brucella melitensis*. J Bacteriol 73, 211-217.

[33] Spink, W. W., Hall, J. W. 3rd., Finstad, J., Mallet, E., 1962. Immunization with viable *Brucella* organisms. Results of a safety test in humans. Bull World Health Organ 26, 409-419.

[34] Jimenez de Bagues, M. P., Barberan, M., Marin, C. M., Blasco, J. M., 1995. The *Brucella abortus* RB51 vaccine does not confer protection against *Brucella ovis* in rams. Vaccine 13, 301-304.

[35] Olsen, S. C., Cheville, N. F., Stevens, M. G., Houng, H. H., Drazek, E. S., Hadfield, T. L., Warren, R. L., Hoover, D. L., 1997. Lymphocyte proliferative responses of goats vaccinated with *Brucella melitensis* 16M or a delta purE201 strain. Infect Immun 65, 2987-2991.

[36] Roop, R. M. 2nd., Jeffers, G., Bagchi, T., Walker, J., Enright, F. M., Schurig, G. G., 1991. Experimental infection of goat fetuses in utero with a stable, rough mutant of *Brucella abortus*. Res Vet Sci 51, 123-127.

[37] Thomas, E. L., Bracewell, C. D., Corbel, M. J., 1981. Characterization of *Brucella abortus* strain 19 cultures isolated from vaccinated cattle. Veterinary Record 108, 90–93.

[38] Sangari, F. J., García-Lobo, J. M., Agüero, J., 1994. The *Brucella abortus* vaccine strain B19 carries a deletion in the erythritol catabolic genes. FEMS Microbiol Lett 121, 337–342.

[39] Mukherjee, F., Jain, J., Grilló, M. J., Blasco, J. M., Nair, M., 2005. Evaluation of *Brucella abortus* S19 vaccine strains by bacteriological tests, molecular analysis of *ery* loci and virulence in BALB/c mice. Biologicals 33, 153–160.

[40] Baldi, P. C., Wallach, J. C., Ferrero, M. C., Delpino, M. V., Fossati, C. A., 2008. Occupational infection due to *Brucella abortus* S19 among workers involved in vaccine production in Argentina. CMI 14, 805–807.

[41] Herr, S., Te Brugge, L. A., 1985. Profiles of serological reactions following adult cow inoculation with standard dose *Brucella abortus* strain 19 vaccine. J S Afr Vet Assoc 56, 93-96.

[42] Nielsen, K., Duncan, J. R., 1988. Antibody isotype response in adult cattle vaccinated with *Brucella abortus* S19. Vet Immunol Immunopathol 19, 205-214.

[43] Mingle, C. K., Manthei, C. A., Jasmin, A. M., 1941. The stability of reduced virulence exhibited by *Brucella abortus*. J Am Vet Med Assoc 99, 203.

[44] Beckett, F. W., Mc Diarmid, S. C., 1985. The effect of reduced-dose *Brucella abortus* strain 19 vaccination in accredited dairy herds. Br Vet J 141, 507-514.

[45] Olsen, S. C., Stoffregen, W. S., 2005. Essential role of vaccines in brucellosis control and eradication programs for livestock. Exp Rev Vac 4, 915–928.

[46] Moriyón, I., Grilló, M. J., Monreal, D., Gonzalez, D., Marin, C., Lopez-Goni, I., Mainar-Jaime, R. C., Moreno, E., Blasco, J. M., 2004. Rough vaccines in animal brucellosis: structural and genetic basis and present status. Vet Res 35, 1–38.

[47] Nicoletti, P., Crowly, A. M., Richardson, J. A., Farrar, J. A., 1986. Suspected *Brucella abortus* strain 19-induced arthritis in a dairy cow. Agri Prac 7, 5-6.

[48] Nicoletti, P., Ring, J., Boysen, B., Buczek, J., 1986. Illness in a veterinary student following accidental inoculation of *Brucella abortus* strain 19. J Am Coll Health 34, 236-237.

[49] Alton, G. G., 1990a. *Brucella melitensis*. In: Animal brucellosis. Nielsen, K. H., Duncan, J. R. (Eds), CRC Press, Boca Raton, 383-409.

[50] Fensterbank, R., Pardon, P., Marly, J., 1982. Efficacy of *Brucella melitensis* Rev. 1 vaccine against *Brucella ovis* infection in rams. Ann Rech Vet 13, 185-190.

[51] Alton, G. G., Elberg, S. S., Crouch, D., 1967. Rev 1 *Brucella melitensis* vaccine. The stability of the degree of attenuation. J Comp Pathol 77, 293-300.

[52] Blasco, J. M., 1997. A review of the use of *B. melitensis* Rev 1 vaccine in adult sheep and goats. Prev Vet Med 31, 275-283.

[53] Munoz, P. M., de Miguel, M. J., Grillo, M. J., Marin, C. M., Barberan, M., Blasco, J. M., 2008. Immunopathological responses and kinetics of *Brucella melitensis* Rev 1 infection after subcutaneous or conjunctival vaccination in rams. Vaccine 26, 2562-2569.

[54] Blasco, J. M., Diaz, R., 1993. *Brucella melitensis* Rev-1 vaccine as a cause of human brucellosis. Lancet 342, 805.

[55] Centers for Disease Control and Prevention (CDC), 1998. Human exposure to *Brucella abortus* strain RB51-Kansas 1997. MMWR Morb Mortal Wkly Rep 47, 172-175.

[56] Schurig, G. G., Roop, R. M., Bagchi, T., Boyle, S., Buhrman, D., Sriranganathan, N., 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 28, 171-188.

[57] Vemulapalli, R., McQuiston, J. R., Schurig, G., Sriranganathan, N., Halling, S. M., Boyle, S. M., 1996. Identification of an *IS711* element interrupting the *wboA* gene of *Brucella abortus* strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. Clin Diagn Lab Immunol 6, 760-764.

[58] Cloeckaert, A., Zygmunt, M. S., Guilloteau, L. A., 2002. *Brucella abortus* vaccine strain RB51 produces low levels of M-like O-antigen. Vaccine 20, 1820-1822.

[59] Stevens, M. G., Hennager, S. G., Olsen, S. C., Cheville, N. F., 1994. Serologic responses in diagnostic tests for brucellosis in cattle vaccinated with *Brucella abortus* 19 or RB51. J Clin Microbiol 32, 1065-1066.

[60] Palmer, M. V., Cheville, N. F., Jensen, A. E., 1996. Experimental infection of pregnant cattle with the vaccine candidate *Brucella abortus* strain RB51: pathologic, bacteriologic, and serologic findings. Vet Pathol 33, 682-691.

[61] Cheville, N. F., Jensen, A. E., Halling, S. M., Tatum, F. M., 1992. Immunology: bacterial survival, lymph node changes, and immunologic responses of cattle vaccinated with standard and mutant strains of *Brucella abortus*. Am J Vet Res 53, 1881-1888.

[62] Palmer, M. V., Olsen, S. C., Cheville, N. F., 1997. Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in pregnant cattle. Am J Vet Res 58, 472-477.

[63] Ashford, D. A., di Pietra, J., Lingappa, J., Woods, C., Noll, H., Neville, B., Weyant, R., Bragg, S. L., Spiegel, R. A., Tappero, J., Perkins, B. A., 2004. Adverse events in humans associated with accidental exposure to the livestock brucellosis vaccine RB51. Vaccine 22, 3435-3439.

[64] Vemulapalli, R., He, Y., Buccolo, L. S., Boyle, S. M., Sriranganathan, N., Schurig, G., 2000. Complementation of *Brucella abortus* RB51 with a functional *wboA* gene results in O-antigen synthesis and enhanced vaccine efficacy but no change in rough phenotype and attenuation. Infect Immun 68, 3927-3932.

[65] Kolar, J., 1989. Brucellosis in eastern European countries. In: Brucellosis: clinical and laboratory aspects. Young, E. J., Corbel, M. J. (Eds.), CRC Press, Boca Raton, chapter 12, 163-172.

[66] Vershilova, P. A., 1961. The use of live vaccine for vaccination of human beings against brucellosis in the USSR. B World Health Organ 24, 85–89.

[67] Lu, S. L., Zhang, J. L., 1989. Brucellosis in China. In: Brucellosis: clinical and laboratory aspects. Young, E. J., Corbel, M. J. (Eds.), CRC Press, Boca Raton, chapter 13, 173-180.

[68] Schurig, G. G., Sriranganathan, N., Corbel, M J., 2002. Brucellosis vaccines: past, present and future. Vet Microbiol 90, 479-496.

[69] Roux, J., Serre, A., 1971. Prophylaxie individuelle et vaccination contre les brucellosis humaines par une fraction antigenique de *Brucella melitensis* (complexe proteins-glycoproteines-glycosaminopeptides). Rev Epidem Med Soc Sante Publ 19, 503-516.

[70] Lopez-Merino, A., Asselineau, J., Serre, A., Roux, J., Bascoul, S., Lacave, C., 1976. Immunization by an insoluble fraction extracted from *Brucella melitensis*: immunological and chemical characterization of the active substances. Infect Immun 13, 311–321.

[71] Renoux, G., Renoux, M., Tinelli, R., 1973. Phenol water fractions from smooth *Brucella abortus* and *Brucella melitensis*: immunochemical analysis and biological behaviour. J Infect Dis 127, 139–148.

[72] Hadjichristodoulou, C., Voulgaris, P., Toulieres, L., Babalis, T., Manetas, G., Goutziana, G., Kastritis, I., Tselentis, I., 1994. Tolerance of the human brucellosis vaccine and the intradermal reaction test for brucellosis. Eur J Clin Microbiol 13, 129–134.

[73] Jacques, I., Olivier-Bernardin, V., Dubray, G., 1991. Induction of antibody and protective responses in mice by *Brucella* O-polysaccharide-BSA conjugate. Vaccine 9, 896-900.

[74] Dranovskaya, E. A., 1991. New approaches to brucellosis vaccination of people with high risk of infection. In: *Brucella* and brucellosis in man and animals. Tumbay, E., Hilmi, S., Ang, O. (Eds.,), University Press, Imzir, Turkish Microbiol Soc, 87–100.

[75] Corbel, M. J., 2006. Brucellosis in humans and animals. Geneva: World Health Organisation.

[76] Montaraz, J. A., Winter, A. J., Hunter, D. M., Sowa, B. A., Wu, A. M., Adams, L. G., 1986. Protection against *Brucella abortus* in mice with O-polysaccharide-specific monoclonal antibodies. Infect Immun 51, 961–963.

[77] Cloeckaert, A., Jacques, I., Bosseray, N., Limet, J. N., Bowden, R., Dubray, G., Plommet, M., 1991. Protection conferred on mice by monoclonal antibodies directed against outer-membrane-protein antigens of *Brucella*. J Med Microbiol 34, 175–180.

[78] Cloeckaert, A., Jacques, I., de Wergifosse, P., Dubray, G., Limet, J. N., 1992. Protection against *Brucella melitensis* or *Brucella abortus* in mice with immunoglobulin G (IgG), IgA, and IgM monoclonal antibodies specific for a common epitope shared by the *Brucella* A and M smooth lipopolysaccharides. Infect Immun 60, 312–315.

[79] Limet, J. N., Bosseray, N., Garin-Bastuji, B., Dubray, G., Plommet, M., 1989. Humoral immunity in mice mediated by monoclonal antibodies against the A and M antigens of *Brucella*. J Med Microbiol 30, 37–43.

[80] Araya, L. N., Winter, A. J., 1990. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infect Immun 58, 254-256.

[81] Yingst, S., Hoover, D. L., 2003. T cell mediated immunity to brucellosis. Crit Rev Microbiol 29, 313-331.

[82] Rolan, H. G., Tsolis, R. M., 2007. Mice lacking components of adaptive immunity show increased *Brucella abortus virB* mutant colonization. Infect Immun 75, 2965–73.

[83] Copin, R., De Baetselier, P., Carlier, Y., Letesson, J. J., Muraille, E., 2007. MyD88-dependent activation of B220-CD11b+LY-6C+ dendritic cells during *Brucella melitensis* infection. J Immunol 78, 5182–91.

[84] Ko, J., Gendron-Fitzpatrick, A., Ficht, T. A., Splitter, G. A., 2002. Virulence criteria for *Brucella abortus* strains as determined by interferon regulatory factor 1-deficient mice. Infect Immun 70, 7004–12.

[85] Murphy, E. A., Sathiyaseelan, J., Parent, M. A., Zou, B., Baldwin, C. L., 2001. Interferon-gamma is crucial for surviving a *Brucella abortus* infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunol 103, 511–18.

[86] Zhan, Y., Liu, Z., Cheers, C., 1996. Tumor necrosis factor alpha and interleukin-12 contribute to resistance to the intracellular bacterium *Brucella abortus* by different mechanisms. Infect Immun 64, 2782-2986.

[87] Zhan, Y. F., Cheers, C., 1995. Endogenous interleukin-12 is involved in resistance to *Brucella abortus* infection. Infect Immun 63, 1387-1390.

[88] Dudal, S., Turriere, C., Bessoles, S., Fontes, P., Sanchez, F., Liautard, J. P., Lafont, V., 2006. Release of LL-37 by activated human Vgamma9Vdelta2 T cells: a microbicidal weapon against *Brucella suis*. J Immunol 177, 5533–39.

[89] Ko, J., Splitter, G., 2003. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. Clin Microbiol Rev 16, 65-78.

[90] Dornand, J., Lafont, V., Oliaro, J., Terraza, A., Castaneda-Roldan, E., Liautard, J. P., 2004. Impairment of intramacrophagic *Brucella suis* multiplication by human natural killer cells through a contact-dependent mechanism. Infect Immun 72, 2303–11.

[91] Fernandes, D. M., Benson, R., Baldwin, C. L., 1995. Lack of a role for natural killer cells in early control of *Brucella abortus* 2308 infections in mice. Infect Immun 63, 4029–33.

[92] Fernandes, D. M., Baldwin,C. L., 1995. Interleukin-10 downregulates protective immunity to *Brucella abortus*. Infect Immun 63, 1130–33.

[93] Svetic, A., Jian, Y. C., Lu, P., Finkelman, F. D., Gause, W. C., 1993. *Brucella abortus* induces a novel cytokine gene expression pattern characterized by elevated IL-10 and IFN-gamma in $CD4^+$ T cells. Int Immunol 5, 877–83.

[94] Xavier, M. N., Winter, M. G., Spees, A. M., Nguyen, K., Atluri, V. L., Silva, T. M. A., Baumler, A. J., Muller, W., Santos, R. L., Tsolis, R. M., 2013. $CD4^+$ T cell-derived IL-10 promotes *Brucella abortus* persistence via modulation of macrophage function. PLOS Pathog 9, e1003454.

[95] Elzner, P. H., Jacobson, R. H., Nielsen, K. H., Douglas, J. T., Winter, A. J., 1994. BALB/c mice infected with *Brucella abortus* express protracted polyclonal responses of both IgG2a and IgG3 isotypes. Immunol Lett 42, 145-150.

[96] Eze, M. O., Yuan, L., Crawford, R. M., Paranavitana, C. M., Hadfield, T. L., Bhattacharjee, A. K., Warren, R. L., Hoover, D. L., 2000. Effects of opsonization and gamma interferon on growth of *Brucella melitensis* 16M in mouse peritoneal macrophages in vitro. Infect Immun 68, 257-263.

[97] Ficht, T. A., Kahl-Mcdonagh, M. M., Arenas-Gamboa, A. M., Rice-Ficht, A. C., 2009. Brucellosis: the case for live, attenuated vaccines. Vaccine 27, D40-D3.

[98] Sangari, F., Aguero, J., 1991. Mutagenesis of *Brucella abortus*: comparative efficiency of three transposon delivery systems. Microb Pathog 11, 443-446.

[99] Hong, P. C., Tsolis, R. M., Ficht, T. A., 2000. Identification of genes required for chronic persistence of *Brucella abortus* in mice. Infect Immun 68, 4102- 4107.

[100] Wu, Q., Pei, J., Turse, C., Ficht, T. A., 2006. Mariner mutagenesis of *Brucella melitensis* reveals genes with previously uncharacterized roles in virulence and survival. BMC Microbiol 6, 102.

[101] Hoover, D. L., Crawford, R. M., Van De Verg, L. L., Izadjoo, M. J., Bhattacharjee, A. K., Paranavitana, C. M., Warren, R. L., Nikolich, M. P., Hadfield, T. L., 1999. Protection of mice against brucellosis by vaccination with *Brucella melitensis* WR201 (16MDeltapurEK). Infect Immun 67, 5877-5884.

[102] Gonzalez, D., Grillo, M. J., de Miguel, M. J., Ali, T., Arce-Gorvel, V., Delrue, R. M., Conde-Alvarez, R., Munoz, P., Lopez-Goni, I., Iriarte, M., Marin, C. M., Weintraub, A., Widmalm, G., Zygmunt, M., Letesson, J. J., Gorvel, J. P., Blasco, J. M., Moriyon, I., 2008. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLoS One 3, e2760.

[103] Edmonds, M. D., Cloeckaert, A., Elzer, P. H., 2002a. *Brucella* species lacking the major outer membrane protein Omp 25 are attenuated in mice and protect against *Brucella melitensis* and *Brucella ovis.* Vet Microbiol 88, 205-221.

[104] Phillips, R. W., Elzer, P. H., Robertson, G. T., Hagius, S. D., Walker, J. V., Fatemi, M. B., Enright, F. M., Roop, R. M. 2nd., 1997. A *Brucella melitensis* high-temperature-requirement A (*htrA*) deletion mutant is attenuated in goats and protects against abortion. Res Vet Sci 63, 165-167.

[105] Grillo, M. J., Bosseray, N., Blasco, J. M., 2000. In vitro markers and biological activity in mice of seel lot strains and commercial *Brucella melitensis* Rev 1 and *Brucella abortus* B19 vaccines. Biologicals 28, 119-127.

[106] Stevens, M. G., Olsen, S. C., Pugh, G. W. Jr., Brees, D., 1995. Comparison of immune responses and resistance to brucellosis in mice vaccinated with *Brucella abortus* 19 or RB51. Infect Immun 63, 264-270.

[107] Stevens, M. G., Olsen, S. C., Pugh, G. W. Jr., Palmer, M. V., 1994. Immune and pathologic responses in mice infected with *Brucella abortus* 19, RB51, or 2308. Infect Immun 62, 3206-3212.

[108] Van, M. D., Kennedy, G. A., Olsen, S. C., Hansen, G. R., Ewalt, D. R., 1999. Brucellosis induced by RB51 vaccine in a pregnant heifer. J Am Vet Med Assoc 215, 1491-1493.

[109] Sanakkayala, N., Sokolovska, A., Gulani, J., Hogenesch, H., Sriranganathan, N., Boyle, S. M., Schurig, G. G, Vemulapalli, R., 2005. Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant *Brucella abortus* RB51. Clin Diag Lab Immunol 12, 1429-1436.

[110] Datta, S. K., Okamoto, S., Hayashi, T., Shin, S. S., Mihajlov, I., Fermin, A., Guiney, D. G., Fierer, J., Raz, E., 2006. Vaccination with irradiated *Listeria* induces protective T cell immunity. Immunity 25, 143-152.

[111] Magnani, D. M., Harms, J. S., Durward, M. A., Splitter, G. A., 2009. Nondividing but metabolically active gamma-irradiated *Brucella melitensis* is protective against virulent *B. melitensis* challenge in mice. Infect Immun 77, 5181-5189.

[112] Moustafa, D., Garg, V. K., Jain, N., Sriranganathan, N., Vemulapalli, R., 2011. Immunization of mice with gamma-irradiated *Brucella neotomae* and its recombinant strains induces protection against virulent *B. abortus, B. melitensis* and *B. suis* challenge. Vaccine 29, 784-794.

[113] Dabral, N., Lafont, M. M., Sriranganathan, N., Vemulapalli, R., 2014. Oral immunization of mice with gamma-irradiated *Brucella neotomae* induces protection against intraperitoneal and intranasal challenge with virulent *B. abortus* 2308. PLOS ONE 9, e107180.

[114] Blander, J. M., Sander, L. E., 2012. Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. Nat Rev Immunol 12, 215-225.

[115] Sander, L. E., Davis, M. J., Boekschoten, M. V., Amsen, D., Dascher, C. C., Ryffel, B., Swanson, J. A., Muller, M., Blander, J. M. 2011. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. Nature 474, 385-389.

[116] Brockstedt, D. G., Bahjat, K. S., Giedlin, M. A., Liu, W., Leong, M., Luckett, W., Gao, Y., Schnupf, P., Kapadia, D., Castro, G., Lim, J. Y., Sampson-Johannes, A., Herskovits, A. A., Stassinopoulos, A., Bouwer, H. G., Hearst, J. E., Portnoy, D. A., Cook, D. N., Dubensky, T. W. Jr., 2005. Killed but metabolically active microbes: a new vaccine paradigm for eliciting effector T-cell responses and protective immunity. Nat Med 11, 853-860.

[117] Skoble, J., Beaber, J. W., Gao, Yi., Lovchik, J. A., Sower, L. E., Liu, W., Luckett, W., Peterson, J. W., Calendar, R., Portnoy, D. A., Lyons, C. R., Dubensky, T. W., 2009. Killed but metabolically active *Bacillus anthracis* vaccines induce broad and protective immunity against anthrax. Infect Immun 77, 1649-1663.

[118] Lankowski, A. J., Hohmann, E. L., 2007. Killed but metabolically active *Salmonella typhimurium*: Application of a new technology to an old vector. J Infect Dis 195, 1203-1211.

[119] Bruhn, K. W., Birnbaum, R., Haskell, J., Vanchinathan, V., Greger, S., Narayan, R., Chang, P., Tran, T. A., Hickerson, S. M., Beverley, S. M., Wilson, M. E., Craft, N., 2012. Killed but Metabolically Active *Leishmania infantum* as a novel whole-cell vaccine for visceral leishmaniasis. Clin Vac Immunol 19, 490-498.

[120] Houten, V. B., Croteau, D. L., DellaVecchia, M. J., Wang, H., Kisker, C., 2005. 'Close-fitting sleeves': DNA damage recognition by the UvrABC nuclease system. Mutat Res 577, 92-117.

[121] Gurunathan, S., Klinman, D. M., Seder, R. A., 2000. DNA vaccines: immunology, application, and optimization. Annu Rev Immunol 18, 927-974.

[122] Onate, A. A., Cespedes, S., Cabrera, A., Rivers, R., Gonzalez, A., Munoz, C., Folch, H., Andrews, E., 2003. A DNA vaccine encoding Cu, Zn superoxide dismutase of *Brucella abortus* induces protective immunity in BALB/c mice. Infect Immun 71, 4857-4861.

[123] Velikovsky, C. A., Cassataro, J., Giambartolomei, G. H., Goldbaum, F. A., Estein, S., Bowden, R. A., Bruno, L., Fossati, C. A., Spitz, M., 2002. A DNA vaccine encoding lumazine synthase from *Brucella abortus* induces protective immunity in BALB/c mice. Infect Immun 70, 2507-2511.

[124] Cassataro, J., Velikovsky, C. A., Barrera, S., Estein, S. M., Bruno, L., Bowden, R., Pasquevich, K. A., Fossati, C. A., Giambartolomei, G. H., 2005. A DNA vaccine coding for the *Brucella* outer membrane protein 31 confers protection against *B. melitensis* and *B. ovis* infection by eliciting a specific cytotoxic response. Infect Immun 73, 6537-6546.

[125] Luo, D., Ni, B., Li, P., Shi, W., Zhang, S., Han, Y., Mao, L., He, Y., Wu, Y., Wang, X., 2006. Protective immunity elicited by a divalent DNA vaccine encoding both the L7/L12 and Omp16 genes of *Brucella abortus* in BALB/c mice. Infect Immun 74, 2734-2741.

[126] Yu, D. H., Hu, X. D., Cai, H., 2007. A combined DNA vaccine encoding BCSP31, SOD, and L7/L12 confers high protection against *Brucella abortus* 2308 by inducing specific CTL responses. DNA Cell Biol 26, 435-443.

[127] Ahlers, J. D., Dunlop, N., Alling, D. W., Nara, P. L. & Berzofsky, J. A., 1997. Cytokine-in-adjuvant steering of the immune response phenotype to HIV-1 vaccine constructs: GM-CSF and TNFα synergize with IL-12 to enhance induction of CTL. J Immunol 158, 3947–3958.

[128] Scheerlinck, J. Y., 2001. Genetic adjuvants for DNA vaccines. Vaccine 19, 2647–2656.

[129] Ahlers, J. D., Belyakov, I. M., Matsui, S. & Berzofsky, J. A., 2001. Mechanisms of cytokine synergy essential for vaccine protection against viral challenge. Int Immunol 13, 897–908.

[130] Kim, J. J., Ayyavoo, V., Bagarazzi, M. L., Chattergoon, M., Boyer, J. D., Wang, B., Weiner, D. B., 1997. Development of a multicomponent candidate vaccine for HIV-1.Vaccine 15, 879–883.

[131] Berzofsky, J. A., Ahlers, J. D., Belyakov, I. M., 2001. Strategies for designing and optimizing new generation vaccines. Nat Rev Immunol 1, 209-219.

[132] Pun, P. B., Bhatt, A. A., Mohan, T., Kulkarni, S., Paranjape, R., Rao, D. N., 2009. Intranasal administration of peptide antigens of HIV with mucosal adjuvant CpG ODN coentrapped in microparticles enhances the mucosal and systemic immune responses. Int Immunopharmacol 9, 468-477.

[133] Bode, C., Zhao, G., Steinhagen, F., Kinjo, T., Klinman, D. M., 2011. CpG DNA as a vaccine adjuvant. Expert Rev Vaccines 10, 499-511.

[134] Al-Mariri, A., Tibor, A., Mertens, P., De Bolle, X., Michel, P., Godefroid, J., Walravens, K., Letesson, J. J., 2001. Protection of BALB/c mice against *Brucella abortus* 544 challenge by vaccination with bacterioferritin or P39 recombinant proteins with CpG oligodeoxynucleotides as adjuvants. Infect Immun 69, 4816-4822.

[135] Neutra, M. R., Pringault, E., Kraehenbuhl, J.-P., 1996. Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annu Rev Immunol 14, 275-300.

[136] Neutra, M. R., Kozlowski, P. A., 2006. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 6, 148-158.

[137] Levine, M. M., 2000. Immunization against bacterial diseases of the intestine. J Pediatr Gastroenterol Nutr 31, 336-355.

[138] Modlin, J. F., 2004. Poliomyelitis in the United States: the final chapter? JAMA 292, 1749-1751.

[139] Jones, B. D., Ghori, N., Falkow, S., 1994. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J Exp Med 180, 15-23.

[140] Sicinski, P., Rowinski, J., Warchol, J. B., Jarzabek, Z., Gut, W., Szczygiel, B., Bielecki, K., Koch, G., 1990. Poliovirus type 1 enters the human host through intestinal M cells. Gastroenterol 98, 56-58.

[141] Kahl-McDonagh, M. M., Arenas-Gamboa, A. M., Ficht, T. A., 2007. Aerosol infection of BALB/c mice with *Brucella melitensis* and *Brucella abortus* and protective efficacy against aerosol challenge. Infect Immun 75, 4923-4932.

[142] Izadjoo, M. J., Bhattacharjee, A. K., Paranavitana, C. M., Hadfield, T. L., Hoover, D. L., 2004. Oral vaccination with *Brucella melitensis* WR201 protects mice against intranasal challenge with virulent *Brucella melitensis* 16M. Infect Immun 72, 4031-4039.

[143] Surendran, N., Sriranganathan, N., Lawler, H., Boyle, S. M., Hiltbold, E. M., Heid, B., Zimmerman, K., Witonsky, S. G., 2011. Efficacy of vaccination strategies against intranasal challenge with *Brucella abortus* in BALB/c mice. Vaccine 29, 2749-2755.

[144] Cleland, J. L., 1999. Single-administration vaccines: controlled-release technology to mimic repeated immunizations. Trends Biotechnol 17, 25-29.

[145] Yeh, M., Chiang, C., 2004. Inactive *Vibrio cholera* whole-cell vaccine-loaded biodegradable microparticles: in vitro release and oral vaccination. J Microencapsul 21, 91-106.

[146] Gilding, D. K., Reed, A. M., 1979. Biodegradable polymers for use in surgery polyglycolic/poly (lactic acid) homo- and copolymers. Polymer 20, 1459-1464.

[147] Kim, H. K., Chung, H. J., Park, T. G., 2006. Biodegradable polymeric microspheres with “open/closed” pores for sustained release of human growth hormone. J Controlled Release 112, 167-174.

[148] Bramwell, V. W., Perrie, Y., 2005. Particulate delivery systems for vaccines. Crit Rev Ther Drug Carrier Syst 22, 151-214.

[149] Lu, D., Garcia-Contreras, L., Xu, D., Kurtz, S. L., Liu, J., Braunstein, M., McMurray, D. N., Hickey, A. J., 2007. Poly (lactide-co-glycolide) microspheres in respirable sizes enhance an in vitro T cell response to recombinant *Mycobacterium tuberculosis* antigen 85B. Pharm res 24, 1834-1843.

[150] Xiang, S. D., Scholzen, A., Minigo, G., David, C., Apostolopoulos, V., Mottram, P. L., Plebanski, M., 2006. Pathogen recognition and development of particulate vaccines: does size matter? Methods 40, 1-9.

[151] Eldridge, J. H., Gilley, R. M., Staas, J. K., Moldoveanu, Z., Meulbroek, J. A., Tice, T. R., 1989. Biodegradable microspheres: vaccine delivery system for oral immunization. Curr Top Microbiol Immunol 146, 59-66.

[152] Woodrow, K. A., Bennett, K. M., Lo, D. D., 2012. Mucosal vaccine design and delivery. Annu Rev Biomed Eng 14, 17-46.

[153] Jepson, M. A., Clark, M. A., Foster, N., Mason, C. M., Bennett, M. K., Simmons, N. L., Hirst, B. H., 1996. Targeting to intestinal M cells. J Anat 189, 507-516.

[154] Chionh, Y. T., Wee, J. L., Every, A. L., Ng, G. Z., Sutton, P., 2009. M-cell targeting of whole killed bacteria induces protective immunity against gastrointestinal pathogens. Infect Immun 77, 2962–2970.

[155] Giannasca, P. J., Giannasca, K. T., Leichtner, A. M., Neutra, M. R., 1999. Human intestinal M cells display the sialyl Lewis A antigen. Infect Immun 67, 946–953.

[156] Arenas-Gamboa, A. M., Ficht, T. A., Kahl-McDonagh, M. M., Rice-Ficht, A. C., 2008. Immunization with a single dose of a microencapsulated *Brucella melitensis* mutant enhances protection against wild-type challenge. Infect Immun 76, 2448-2455.

[157] Arenas-Gamboa, A. M., Ficht, T. A., Davis, D. S., Elzer, P. H., Kahl-McDonagh, M., Wong-Gonzalez, A., Rice-Ficht, A. C., 2009. Oral vaccination with microencapsulated strain 19 vaccine confes enhanced protection against *Brucella abortus* strain 2308 challenge in red deer (*Cervus elaphus elaphus*). J Wildl Dis 45, 1021-1029.

[158] Arenas-Gamboa, A. M., Ficht, T. A., Davis, D. S., Elzer, P. H., Wong-Gonzalez, A., Rice-Ficht, A. C., 2009. Enhanced immune response of red deer (*Cervus elaphus*) to live RB51 vaccine strain using composite microspheres. J Wildl Dis 45, 165-173.

[159] Arenas-Gamboa, A. M., Ficht, T. A., Kahl-McDonagh, M., Gomez, G., Rice-Ficht, A. C., 2009. The *Brucella abortus* S19 DeltavjbR live vaccine candidate is safer than S19 and confers protection against wild-type challenge in BALB/c mice when delivered in sustained-release vehicles. Infect Immun 77, 877-884.

[160] Arenas-Gamboa, A. M., Ficht, T. A., Kahl-McDonagh, M., Rice-Ficht, A. C., 2008. Immunization with a single dose of a microencapsulated *Brucella melitensis* mutant enhances protection against wild-type challenge. Infect Immun 76, 2448-2455.

[161] Vemulapalli, R., Contreras, A., Sanakkayala, N., Sriranganathan, N., Boyle, S. M., Schurig, G. G., 2004. Enhanced efficacy of recombinant *Brucella abortus* RB51 vaccines against *B. melitensis* infection in mice. Vet Microbiol 102, 237-245.

[162] Gil, Y. R., Iriarte, M., C., Moriyon, I. U., 2012. Diva method for differentiating animals vaccinated against brucellosis. WO 2012141128 A1.

CHAPTER 3. GAMMA IRRADIATION: REPURPOSING AN OLD TOOL FOR DEVELOPING SAFER VACCINES AGAINST INTRACELLULAR PATHOGENS

Abstract

Successful vaccination strategies attempt to mimic natural infection for induction of long lasting immunity against infectious diseases. An effective vaccine should stimulate both antibody-mediated and cell-mediated immune responses that are required to eliminate the pathogen from the body. Observations till date indicate that vaccines containing live organisms are far superior to the killed ones in inducing protective immunity. Live vaccines induce a broad, robust cell-mediated immune response and a long-lasting immunological memory. In fact, only live vaccine are effective against some infectious diseases such as tuberculosis and brucellosis, which are caused by intracellular bacterial pathogens. However, the replication potential of organisms in live vaccines poses a safety risk, at least to some individuals in a population. Exposure to ionizing radiation has been used to generate non-replicative but metabolically active microorganisms. While possessing the safety profile of killed vaccines, irradiated vaccines retain sufficient metabolic activity to initiate potent immune responses similar to that of live vaccines. This review focuses on gamma-irradiation and its applicability as a promising technique for development of safer and efficacious vaccines against intracellular pathogens.

Introduction

Vaccination is the most efficient medical intervention employed till to date to reduce infectious disease burden in animals and humans [1]. Since the advent of vaccination, inactivation and attenuation of pathogens have been the most widely used strategies for development of vaccines. Undoubtedly, live attenuated vaccines are more immunogenic and induce longer lasting protective responses than vaccines based on inactivated forms. However, there are inherent safety risks associated with the use of live vaccines; they often retain some pathogenicity and can also revert back to being fully virulent. Empirical attenuation, in instances where the molecular basis of attenuation is not known, is often unreliable. Even in cases of rational attenuation, the live attenuated vaccines can cause serious adverse effects, especially in immunocompromised subjects [2]. In contrast, inactivated vaccines, although reliably safe, induce a weaker immunity that often does not translate to long-lasting protection. Developing vaccines that are efficacious and yet possess the safety profile of killed vaccines is highly desirable.

Ionizing radiation, such as X-rays and gamma-rays, can cause damage to cellular components, and at sufficiently high enough doses can cause cell death. Gamma-rays have higher energy than X-rays. In aqueous conditions of biological materials, ionizing radiation causes formation of reactive oxygen species (ROS) from water, and ROS cause the damaging effects on DNA, proteins, and other cellular components. Exposure to gamma-rays (gamma-irradiation) is a widely used technique for inactivation of pathogens in food to avoid spoilage [3], sterilization of pharmaceuticals [4,5], medical/research supplies and tissue/biological based products [6]. The Center for Biologics Evaluation and Research of the United States Food and Drug Administration has approved gamma-irradiation for sterilization of plastic containers and diluents used in vaccine manufacturing. Using ionizing radiation for developing vaccines dates back to 1936, when Moore and Kersten reported the use of X-rays to inactivate *Shigella dysenteriae* [7]. Remarkably, studies conducted in 1950s with X-ray irradiation led to the development of a commercial vaccine (Dictol) against *Dictyocaulus viviparous*, a nematode parasite of cattle. The third stage larvae of *D. viviparous* were subjected to an optimal dose of X-rays

such that their development halted at the fourth larval stage and they did not form adult worms. Vaccination with the irradiated larvae induced host immunity and conferred protection to cattle [8]. Since then, gamma-rays and other ionizing radiations have been used for the development of viral, bacterial and parasitic vaccines [9-15]; however, the specific benefits of irradiation in the development of safer vaccines for intracellular pathogens are yet to be fully explored. Thirty six years ago, gamma-irradiation as a technique for vaccine inactivation was illustrated in a murine model of *Rickettsia* [16,17]. The findings established that irradiated scrub typhus rickettsiae induced protection in mice which was superior to that of formalin-killed vaccine. Subsequently, many studies have demonstrated the use of gamma-rays for development of effective and safer vaccines. The focus of this chapter is limited to discussing the benefits of gamma-irradiation as a technique for developing safer vaccines, especially for intracellular pathogens.

Immune sensing of pathogen metabolic activity

The mammalian immune system has innate and adaptive components; together they orchestrate to protect the host against microbial infections. Innate immune responses influence the development of subsequent adaptive immunity to the pathogens through recruitment and activation of antigen presenting cells (APCs).

APCs express pattern-recognition receptors (PRRs) which can bind with distinct microbial components that are unique to microorganisms, often referred to as pathogen-associated molecular patterns (PAMPs). The best known PRRs are Toll-like receptors (TLRs), but a number of other cell surface and intracellular receptors are also involved in sensing the presence of PAMPs. [18,19]. The rapid and effective sensing of PAMPs stimulates the host immune responses via activation of complex signaling pathways leading to an inflammatory response that subsequently assists in pathogen clearance [20]. Both live and killed vaccines contain PAMPs (Table 3.1). However, live attenuated vaccines like typhoid or tuberculosis vaccine induce a potent immune response that leads to long-lasting protection when compared with vaccines that are based on killed organisms (Table 3.1). This suggests that the host innate defense can sense the presence of live microorganisms

and launch a more robust immune response against them. A recent study has demonstrated that the host immune mechanisms can make a fine distinction between live and dead microorganisms by sensing their viability itself [21].

The host immune system perceives a vaccine as being ‘live’ or ‘viable’ only if the vaccine contains viability-associated PAMPs (vita-PAMPs) which are present in live microbes. In case of bacteria, bacterial messenger RNA (mRNA) are an example of vita-PAMPs associated with live bacteria [21]. Products of microbial metabolism such as bacterial pyrophosphates and second messengers such as cyclic di-GMP are other potential vita-PAMPs that help the host system to differentiate between live and killed microbes [22]. Recognition of vita-PAMPs leads to inflammasome activation and pyroptosis. Previous reports have documented that replication-unfit ‘killed-but-metabolically-active’ (KBMA) bacteria are able to mount a robust immune response indicating that bacterial metabolic activity by itself may be sufficient to produce vita-PAMPs [23].

Several previous studies reported that gamma-ray attenuated bacteria and parasites retain their metabolic activity, though they lose their ability to replicate [11-13,24]. Gamma-irradiation leads to the generation of ROS that cause random double-strand disruptions in the DNA [25,26]. The extent of the resulting DNA damage is dependent on the genome size of the organism and is inversely related to the dose of the gamma-rays [27,28]. Exposure of vaccine organism to a minimum dose of gamma-radiation that causes sufficient DNA fragmentation and abrogation of its replication capacity is an attractive strategy for developing effective vaccines. Sufficient DNA fragmentation can overwhelm the DNA repair machinery of the bacteria and abolish their ability to replicate but leave an ample portion of the genome still intact to permit the organism to be metabolically active and express genes de novo and consequently, synthesize proteins [11,13]. This would help facilitate the recognition, processing and presentation of the antigenic repertoire by the host defense system leading to the induction of protective immunity at levels comparable to live vaccines. In addition to irradiation, other approaches based on genetic manipulation of the organism were used for developing non-replicative but metabolically active vaccines [29-32]. Such vaccines were also demonstrated to be effective in inducing robust immune responses on par with live vaccines. Taken together, these findings suggest that retention

of metabolic activity by the vaccine organisms is crucial for induction of effective immunity. Future studies should focus on demonstrating the role of vita-PAMP recognition in immunity induced by gamma-irradiated vaccines.

Maintaining structural, functional and epitope integrity

Unlike inactivation by heat or chemical treatment, gamma-irradiated organisms remain structurally intact. Gamma-irradiation at a dose used in vaccine preparation does not cause denaturation of structures and proteins. Structural integrity of the functional domains of the microbe is crucial for effective antigen processing and the development of immunity against natural infections. For example, gamma-ray inactivation of pathogenic viruses like Lassa, Marburg and Ebola, renders the microbes non-infectious without affecting their immunological activity [33]. Gamma-irradiated viruses retain the ability to induce cytotoxic T cells [34-36]. These findings suggest that the irradiated virus can infect target host cells, allowing natural and efficient uptake of antigens by APCs and major histocompatibility complex I (MHC I) presentation of viral antigens for the induction of cell-mediated immunity. Chemical inactivation methods, on the contrary, cause extensive cross-linking of the microbial proteins [37], thereby compromising the epitope structure as well as functionality. Gamma-irradiated *Brucella* do not lose their structural integrity even after 48 hours of infection of macrophage cells [13] indicating that gamma-rays have no residual adverse effect on the microbe structure, for at least upto 2 days post-infection.

Gamma-irradiated organisms also remain functionally intact. For example, gamma-irradiated *Listeria* retains the ability to escape into the cytosolic compartment and induce robust immunity [12]. This is only possible if the gamma-irradiated *Listeria* expresses listeriolysin O which mediates its escape into the cytosol. Similarly, gamma-irradiated *Brucella* is able to direct its intracellular trafficking in a way to avoid degradation within phagolysosomes [13]. This indicates that, while inside the macrophages, gamma-irradiated *Brucella* can express proteins that are necessary for its modulation of the endocytic pathway and its survival. Gamma-irradiated *Toxoplasma gondii* is capable of infecting cells, thereby inducing robust Th-1 biased immune responses. It exhibits intact ability to

synthesize proteins and even nucleic acids [38]. Hence, gamma-irradiated organisms are able to express effectors that allow them to mimic the infection pattern of live pathogens and influence the effectiveness of the elicited immune responses. This intact transcriptional and translational activity confers enhanced immunogenicity to the gamma-irradiated vaccines.

Inducing protective immune responses

Gamma-irradiated vaccines are capable of eliciting protection in vaccinated hosts (Table 3.2). Attenuation by gamma-rays has been used for the development of a cross-protective experimental vaccine against influenza. The most commonly used influenza vaccines are trivalent split or subunit vaccines that are strain-matched to the seasonal circulating strains. These vaccines rely on the induction of strain-specific neutralizing antibodies. In contrast to the strain-matched influenza vaccines, natural influenza infections induce heterosubtypic immunity. Protection against heterosubtypic strains has been attributed mainly to the development of cross-protective cytotoxic T cells against the virus-infected cells. Generally, whole virus based vaccines are more immunogenic against influenza than subunit or split-product vaccines which frequently require adjuvants and/or multiple immunization regimens. Whole virus based vaccines activate the innate immune system through TLR-7 which leads to Th1-biased favorable immune responses. Polley et. al. first demonstrated the induction of serum neutralizing antibodies and protection against homotypic strains by intra-peritoneal (i.p.) vaccination with a gamma-irradiated H1N1 virus [39]. Subsequently, many reports confirmed the ability of gamma-irradiated viruses to afford homologous protection [40,41]. The cross-protective potential of gamma-irradiated influenza vaccines was first demonstrated by Mullbacher et. al. [36]. These cross protective responses are believed to be mainly directed against the internal proteins, such as the nucleoprotein, which are conserved among the influenza A strains [42]. Interestingly, equivalent doses of formaldehyde treated or ultraviolet (UV) irradiated whole inactivated virus preparations failed to induce cross-protective cytotoxic T cell responses [43]. Recently, a study showed that a single intra-nasal (i.n.) administration of gamma-irradiated

H1N1 virus afforded protection against a lethal H5N1 avian influenza virus challenge and other heterosubtypic influenza A infections [44]. Gamma Vaccines Pty Ltd, Australia, is currently working towards the development of a universal gamma-irradiated influenza vaccine (GammaFlu^{TM}) that would target both seasonal as well as pandemic markets. Unlike with the live-attenuated vaccines, there is no risk of reassortment with the newly emerging strains and reverting to a virulent form with gamma-irradiated influenza virus vaccines.

Radiation attenuation has also contributed significantly to the efforts of developing a vaccine against malaria. Since the 1970s, researchers have studied vaccination by the bite of mosquitoes containing radiation-attenuated sporozoites of *Plasmodium falciparum* in their salivary glands [45-48]. A recent study reported that this type of vaccination induced protection in more than 90% of the human volunteers [49].

Conclusions and future directions:

Till date, vaccination strategies, particularly against intracellular microorganisms, have delivered limited success. Regarding vaccine research, the bottleneck is likely to lie in the extensive regulatory restrictions which prevent the translation of promising vaccine research from preclinical trials to the relevant subjects on account of safety or efficacy concerns. This translational block can be overcome by using techniques which render the vaccinal microorganisms permanently non-replicative but still capable of inducing high levels of immunity. Subjecting the vaccine to an optimum dose of gamma-irradiation assures non-reversion to virulence and, consequently, the research is more likely to ease into the phase of clinical investigation to establish the vaccine efficacy. In contrast to rational attenuation, gamma-irradiation is an easily applicable method for broadly inactivating a large batch of vaccine preparation. It is likely that using gamma-irradiation will allow the development of a more effective and safer alternative to the classic-attenuated and killed-pathogen vaccine, especially for chronic infections caused by intracellular microorganisms. Gamma-irradiation can also be used for the rapid production of safer vaccines in-bulk for emerging pathogens, such as new strains of influenza viruses

and Ebola virus. The process of gamma-irradiation also eliminates the purification steps and other cumbersome procedural drawbacks associated with inactivation methods that employ chemicals. Moreover, it is a relatively inexpensive process that can be an attractive and indispensable alternative for nations with limited resources. However, further research is needed to establish the shelf-life and thermal stability of irradiated vaccines. Gamma-irradiation can also be combined with vaccine adjuvants and targeted molecular strategies, such as deleterious gene mutations or inclusion of additional genes, in order to obtain the desired immunogenic priming and enhanced protective immunity.

References

[1] WHO, 2005. Immunization against diseases of public health importance. In Fact Sheets 288.

[2] L'Huillier, A. G., Posfay-Barbe, K. M., 2014. Live viral vaccines in immunocompromised patients. Fut Microbiol 9, 161-171.

[3] WHO, 1994. Safety and nutritional adequacy of irradiated food. WHO Report 161.

[4] Sintzel, M. B., Merkli, A., Tabatabay, C., Gurny, R., 1997. Influenze of irradiation sterilization on polymers used as drug carriers- a review. Drug Development and Industrial Pharmacy 23, 857-878.

[5] Marciniec, B., Dettlaff, K., 2008. Radiation sterilization of drugs. In: Trends in Radiation Sterilization of Health Care Products. Haji-Saeid, M., (Ed.), International Atomic Energy Agency, Vienna, 187–230.

[6] Dziedzic-Goclawska, A., Kaminski, A., Uhrynowska-Tyszkiewicz, I., Michalik, J., Stachowicz, W., 2008. Radiation sterilization of human tissue grafts. In: Trends in Radiation Sterilization of Health Care Products. Haji-Saeid, M., (Ed.), International Atomic Energy Agency, Vienna, 231–260.

[7] Moore, H. N., Kersten, H., 1936. Preliminary note on the preparation of non-toxic Shiga dysentery vaccines by irradiation with soft X-rays. J Bacteriol 31, 581-584.

[8] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., Urquhart, G. M., 1960. Immunological studies on *Dictyocaulus viviparus* infection: immunity produced by the administration of irradiated larvae. Immunol 3, 145-151.

[9] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., Urquhart, G. M., 1965. Irradiated helminth larvae in vaccination. Proc R Soc Med 51, 743-744.

[10] Hubbert, W. T., Miller, J. N., 1965. Studies on immunity in experimental leptospirosis: the immunogenicity of *Leptospira icterohemorrhagiae* attenuated by gamma-irradiation. J Immunol 95, 759-764.

[11] Sanakkayala, N., Sokolovska, A., Gulani, J., Hogenesch, H., Sriranganathan, N., Boyle, S. M., Schurig, G. G, Vemulapalli, R., 2005. Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant *Brucella abortus* RB51. Clin Diag Lab Immunol 12, 1429-1436.

[12] Datta, S. K., Okamoto, S., Hayashi, T., Shin, S. S., Mihajlov, I., Fermin, A., Guiney, D. G., Fierer, J., Raz, E., 2006. Vaccination with irradiated *Listeria* induces protective T cell immunity. Immunity 25, 143-152.

[13] Magnani, D. M., Harms, J. S., Durward, M. A., Splitter, G. A., 2009. Nondividing but metabolically active gamma-irradiated *Brucella melitensis* is protective against virulent *B. melitensis* challenge in mice. Infect Immun 77, 5181-5189.

[14] Martin, S. S., Bakken, R. R., Lind, C. M., Garcia, P., Jenkins, E., Glass, P. J., Parker, M. D., Hart, M. K., Fine, D. L., 2010. Comparison of the immunological responses and efficacy of gamma-irradiated V3526 vaccine formulations against subcutaneous and aerosol challenge with Venezuelan equine encephalitis virus subtype IAB. Vaccine 28, 1031-1040.

[15] Bordin, A. I., Pillai, S. D., Brake, C., Bagley, K. B., Bourquin, J. R., Coleman, M., Oliveira, F. N., Mwangi, W., McMurray, D. N., Love, C. C., Felippe, M. J. B., Cohen, N. D., 2014. Immunogenicity of an electron beam inactivated *Rhodococcus equi* vaccine in neonatal foals. PLOS ONE 9, e105367.

[16] Eisenberg, G. H. Jr., Osterman, J. V, 1978. Gamma-irradiated scrub typhus immunogens: development and duration of immunity. Infect Immun 22, 80-86.

[17] Eisenberg, G. H. Jr., Osterman, J. V., 1979. Gamma-irradiated scrub typhus immunogens: broad-spectrum immunity with combinations of rickettsial strains. Infect Immun 26, 131-136.

[18] Kumar, H., Kawai, T., Akira, S., 2011. Pathogen recognition by the innate immune system. Int Rev Immunol 30, 16-34.

[19] Vance, R. E., Isberg, R. R., Portnoy, D. A., 2009. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. Cell Host Microbe 6, 10-21.

[20] Medzhitov, R., 2007. Recognition of microorganisms and activation of the immune response. Nature 449, 819-826.

[21] Sander, L. E., Davis, M. J., Boekschoten, M. V., Amsen, D., Dascher, C. C., Ryffel, B., Swanson, J. A., Muller, M., Blander, J. M. 2011. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. Nature 474, 385-389.

[22] Blander, J. M., Sander, L. E., 2012. Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. Nat Rev Immunol 12, 215-225.

[23] Dubensky, T. W., Skoble, J., Lauer, P., Brockstedt, D. G., 2012. Killed but metabolically active vaccines. Curr Opin Biotechnol 23, 917-923.

[24] Moustafa, D., Garg, V. K., Jain, N., Sriranganathan, N., Vemulapalli, R., 2011. Immunization of mice with gamma-irradiated *Brucella neotomae* and its recombinant strains induces protection against virulent *B. abortus, B. melitensis* and *B. suis* challenge. Vaccine 29, 784-794.

[25] Henner, W. D., Grunberg, S. M., Haseltine, W. A., 1982. Sites and structure of gamma radiation-induced DNA strand breaks. J Biol Chem 257, 11750-11754.

[26] Trampuz, A., Piper, K. E., Steckelberg, J. M., Patel, R., 2006. Effect of gamma irradiation on viability and DNA of *Staphylococcus epidermidis* and *Escherichia coli*. J Med Microbiol 55, 1271-1275.

[27] Ginoza, W., 1968. Inactivation of viruses by ionizing radiation and by heat. In: Methods in Virology. Mararorosch, K., Koprowski, H., (Eds.), Vol 4, Academic Press, NY, 139-209.

[28] Rohwer, R. G., 1984. Scrapie infectious agent is virus-like in size and susceptibility to inactivation. Nature 308, 658-662.

[29] Brockstedt, D. G., Bahjat, K. S., Giedlin, M. A., Liu, W., Leong, M., Luckett, W., Gao, Y., Schnupf, P., Kapadia, D., Castro, G., Lim, J. Y., Sampson-Johannes, A., Herskovits, A. A., Stassinopoulos, A., Bouwer, H. G., Hearst, J. E., Portnoy, D. A., Cook, D. N., Dubensky, T. W. Jr., 2005. Killed but metabolically active microbes: a new vaccine paradigm for eliciting effector T-cell responses and protective immunity. Nat Med 11, 853-860.

[30] Skoble, J., Beaber, J. W., Gao, Yi., Lovchik, J. A., Sower, L. E., Liu, W., Luckett, W., Peterson, J. W., Calendar, R., Portnoy, D. A., Lyons, C. R., Dubensky, T. W., 2009. Killed but metabolically active *Bacillus anthracis* vaccines induce broad and protective immunity against anthrax. Infect Immun 77, 1649-1663.

[31] Lankowski, A. J., Hohmann, E. L., 2007. Killed but metabolically active *Salmonella typhimurium*: Application of a new technology to an old vector. J Infect Dis 195, 1203-1211.

[32] Bruhn, K. W., Birnbaum, R., Haskell, J., Vanchinathan, V., Greger, S., Narayan, R., Chang, P., Tran, T. A., Hickerson, S. M., Beverley, S. M., Wilson, M. E., Craft, N., 2012. Killed but Metabolically Active *Leishmania infantum* as a novel whole-cell vaccine for visceral leishmaniasis. Clin Vac Immunol 19, 490-498.

[33] Elliott, L. H., McCormick, J. B., Johnson, K. M., 1982. Inactivation of Lassa, Marburg, and Ebola viruses by gamma irradiation. J Clin Microbiol 16, 704-708.

[34] Mullbacher, A., Ada, G. L., Hla, R. T., 1988. Gamma-irradiated influenza A virus can prime for a cross-reactive and cross-protective immune response against influenza A viruses. Immunol Cell Biol 66, 153-157.

[35] Müllbacher, A., Marshall, I. D., Blanden, R. V., 1979. Cross-reactive cytotoxic T cells to alphavirus infection. Scand J Immunol 10, 291–296.

[36] Müllbacher, A., Marshall, I. D., Ferris, P., 1986. Classification of Barmah Forest virus as an alphavirus using cytotoxic T cell assays. J Gen Virol 67, 295–299.

[37] Jackson, V., 1978. Studies on histone organization in the nucleosome using formaldehyde as a reversible cross-linking agent. Cell 15, 945-954.

[38] Hiramoto, R. M., Galisteo, A. J., do Nascimento, N., de Andrade, H. F. Jr., 2002. 200 Gy sterilised *Toxoplasma gondii* tachyzoites maintain metabolic functions and mammalian cell invasion, eliciting cellular immunity and cytokine response similar to natural infection in mice. Vaccine 20, 2072-2081.

[39] Polley, J. R., 1961. Factors influencing inactivation of infectivity and hemagglutinin of influenza virus by gamma radiation. Can J Microbiol 7, 535–541.

[40] Kulevich, E. E., Kosiakov, P. N., 1974. Immunogenic properties of A2/Hongkong/1/68 influenza virus inactivated with gamma rays. Vopr Virusol 6, 696–700.

[41] Noack, K., Tischner, H., Brauniger, S., Nordheim, W., Pohl, W. D., 1986. Long-term effect of oral immunization against influenza with a gamma ray-inactivated vaccine in mice. Z Erkr Atmungsorgane 166, 286–289.

[42] Epstein, S. L., Kong, W. P., Misplon, J. A., Lo, C. Y., Tumpey, T. M., Xu, L., Nabel, G. J., 2005. Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. Vaccine 23, 5404-5410.

[43] Furuya, Y., Regner, M., Lobigs, M., Koskinen, A., Mullbacher, A., Alsharifi, M., 2010. Effect of inactivation method on the cross-protective immunity induced by whole 'killed' influenza A viruses and commercial vaccine preparations. J Gen Virol 91, 1450-1460.

[44] Alsharifi, M., Furuya, Y., Bowden, T. R., Lobigs, M., Koskinen, A., Regner, M., Trinidad, L., Boyle, D. B., Müllbacher, A., 2009. Intranasal flu vaccine protective against seasonal and H5N1 avian influenza infections. PLOS ONE 4, e5336.

[45] Clyde, D. F., McCarthy, V. C., Miller, R. M., Woodward, W. E., 1975. Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. Am J Trop Med Hyg 24, 397-401.

[46] Clyde, D. F., 1990. Immunity to falciparum and vivax malaria induced by irradiated sporozoites: a review of the University of Maryland studies, 1971–75. Bull World Health Organ 68, 9-12.

[47] Rieckmann, K. H., Beaudoin, R. L., Cassells. J. S., Sell, D. W., 1979. Use of attenuated sporozoites in the immunization of human volunteers against falciparum malaria. Bull World Health Organ 57, 261-5.

[48] Rieckmann, K. H., 1990. Human immunization with attenuated sporozoites. Bull World Health Organ 68, 13-6.

[49] Hoffman, S. L., Goh, L. M., Luke, T. C., Schneider, I., Le, T. P., Doolan, D. L., Sacci, J., Vega, P. de L., Dowler, M., Paul, C., Gordon, D. M., Stoute, J. A., Church, L. W., Sedegah, M., Heppner, D. G., Ballou, W. R., Richie, T. L., 2002. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. J Infect Dis 185, 1155-1164.

[50] Begum, R. E., Rahman, H., Ahmed, G., 2011. Development and evaluation of gamma irradiated toxoid vaccine of *Salmonella enterica* var Typhimurium. Vet Microbiol 153, 191-197.

[51] Younis, S. A., Yagi, A. I., Haroun, E. M., Gameel, A. A., Taylor, M. G., 1986. Immunization of zebu calves against *Fasciola gigantica*, using irradiated metacercariae. J Helminthol 60, 123–134.

[52] Ramamoorthy, S., Sanakkayala, N., Vemulapalli, R., Neeta, J., Lindsay, D. S., Schurig, G. S., Boyle, S. M., Sriranganathan, N., 2007. Prevention of vertical transmission of *Neospora caninum* in C57BL/6 mice vaccinated with *Brucella abortus* strain RB51 expressing *N. caninum* protective antigens. Int J Parasitol 37, 1531–1538.

[53] Bickle, Q. D., Bøgh, H. O., Johansen, M. V., Zhang, Y., 2001. Comparison of the vaccine efficacy of gamma-irradiated *Schistosoma japonicum* cercariae with the defined antigen Sj62 (IrV-5) in pigs. Vet Parasitol 100, 51-62.

[54] Samantaray, S. N., Bhattacharyulu, Y., Gill, B. S., 1980. Immunization of calves against bovine tropical theileriosis (*Theileria annulata*) with graded doses of sporozoites and irradiated sporozoites. Int J Parasitol 10, 355–358.

[55] Morrison, W. I., Black, S. J., Paris, J., Hinson, C. A., Wells, P. W., 1982. Protective immunity and specificity of antibody responses elicited in cattle by irradiated *Trypanosoma brucei*. Parasite Immunol 4, 395–407.

[56] Campbell, C. H., Barber, T. I., Knudson, R. C., Swaney, L. M., 1985. Immune response of mice and sheep to bluetongue virus inactivated by gamma irradiation. Prog Clin Biol Res 178, 639 - 647.

Table 3.1. Differences between killed, gamma-irradiated and live vaccines.

	Killed vaccines	Gamma-irradiated vaccines	Live vaccines
PAMPs	✓	✓	✓
vita-PAMPs	X	✓	✓
Replication	X	X	✓
Reversion to virulence	X	X	✓
Gene expression	X	✓	✓
Immunogenicity	weaker	stronger	stronger
Duration of immunity	short lasting	long lasting	long lasting
Type of immunity	mostly antibody mediated	antibody and cell mediated	antibody and cell mediated
Adjuvant	required	not required	not required
Stability	good	good	requires constant refrigeration

Table 3.2. Induction of antigen-specific immune responses and protection by gamma-irradiated vaccines in the indicated host species.

Name of vaccinal organism	Gamma-irradiation dose (Gy)*	Host species	Antigen specific immune responses	Protective response	Reference
		Bacterial vaccines			
Brucella abortus RB51	3000	Mice	IgG, IgG2a, IFN- γ	Vaccinated mice showed a 0.85 log reduction in splenic bacterial load following challenge with virulent *Brucella* spp.	[11]
Brucella melitensis	3500	Mice	Cytotoxic T cells	1 log reduction in bacterial load in the spleen was observed against challenge with virulent *Brucella* spp.	[13]
Brucella neotomae	3500	Mice	IgG1, IgG2a, IgG2b, IgG3, IFN-γ secreting $CD4^+$ and $CD8^+$ T cells	Vaccinated mice showed a 2-3 log reduction in bacterial load in the spleen following challenge with virulent *Brucella* spp.	[24]

Leptospira icterohemorrhagiae	500	Guinea pigs	No information	92% of the vaccinated guinea pigs were protected against infection.	[10]
Listeria monocytogenes	6000	Mice	IFN-γ secreting $CD4^+$ and $CD8^+$ T cells	Vaccinated mice showed a 2 log decrease in splenic and liver bacterial load and 80% vaccinated animals were protected against lethal challenge.	[12]
Salmonella enterica var Typhimurium toxoid	40,000	Kuroiler birds	No information	100% birds were protected against homologous challenge. 100% and 83% birds were protected against challenge with heterologous strain through oral and intra-peritoneal routes, respectively.	[50]

		Parasitic vaccines			
Fasciola gigantica metacercariae	200	Calves	No information	88% reduction in worm burden when challenged with 1000 cercariae.	[51]
Neospora caninum	528	Mice	IgG1, IgG2a, IFN-γ, IL-10	100% protection was observed against lethal challenge infection.	[52]
Plasmodium falciparum sporozoites	150	Humans	No information	More than 90% humans protected against Malaria	[49]
Schistosoma japonicum	200	Pigs	IgG	More than 95% resistance to infection was observed.	[53]
Theileria annulata sporozoites	100-150	Calves	No information	100% calves protected against pathogenic challenge.	[54]
Toxoplasma gondii	200	Mice	IgG and IFN-γ	Vaccinated mice survived longer than naturally infected control mice.	[38]

Trypanosoma brucei	600	Cattle	IgG and IgM	Vaccination with 10^7 irradiated trypanosomes protected against challenge with 10^3 homologous trypanosomes	[55]
Viral vaccines					
Blue Tongue virus	60000	Sheep	Neutralizing antibodies	Vaccinated sheep were protected against infection.	[56]
Influenza virus	10,000	Mice	Neutralizing antibodies against homologous strain, cross-reactive cytotoxic T cells	Vaccinated mice were protected against homologous and heterosubtypic challenges, including challenge with a H5N1 avian virus strain.	[34] and [44]
Venezuelan equine encephalitis virus	50,000	Mice	Neutralizing antibodies	More than 80% of the vaccinated animals were protected against a subcutaneous challenge.	[14]

*Gy (Gray) indicates the SI derived unit of absorbed dose of gamma rays.

CHAPTER 4. OVEREXPRESSION OF *BRUCELLA* PUTATIVE GLYCOSYLTRANSFERASE WBKA IN *B. ABORTUS* RB51 LEADS TO PRODUCTION OF EXOPOLYSACCHARIDE

Abstract

Brucella spp. are Gram-negative, facultative intracellular bacteria that cause brucellosis in mammals. *Brucella* strains containing the O-polysaccharide in their cell wall structure exhibit a smooth phenotype whereas the strains devoid of the polysaccharide show rough phenotype. *B. abortus* strain RB51 is a stable rough attenuated mutant which is used as a licensed live vaccine for bovine brucellosis. Previous studies have shown that the *wboA* gene, which encodes a glycosyltransferase required for the synthesis of O-polysaccharide, is disrupted in *B. abortus* RB51 by an *IS711* element. Although complementation of strain RB51 with a functional *wboA* gene results in O-polysaccharide synthesis in the cytoplasm, it does not result in a smooth phenotype. The aim of this study was to determine if overexpression of *Brucella* WbkA or WbkE, putative glycosyltransferases essential for O-polysaccharide synthesis, in strain RB51 would result in the O-polysaccharide synthesis and smooth phenotype. Here, we demonstrate that overexpression of *wbkA* or *wbkE* gene in RB51 did not result in the O-polysaccharide expression as shown by Western blotting with specific antibodies. However, *wbkA*, but not *wbkE*, overexpression led to the development of a clumping phenotype and the production of exopolysaccharide(s) containing mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine. Moreover, we found that the clumping recombinant strain displayed increased adhesion to

polystyrene plates. The recombinant strain was similar to strain RB51 in its attenuation characteristic and in its ability to induce protective immunity against virulent *B. abortus* challenge in mice.

Introduction

Members of the genus *Brucella* are Gram-negative, facultative intracellular coccobacilli that can cause chronic infections in several mammals, including humans. Based on the structure of the lipopolysaccharide (LPS) molecule and the colony morphology, *Brucella* spp. can be separated into smooth and rough phenotypes. Smooth colony morphology of *Brucella* strains is determined by the presence of LPS containing the O-polysaccharide (O-PS) in their cell wall structure. *Brucella* spp. with LPS that is devoid of the O-PS display rough colony morphology. Based on the reactive specificities of antibodies, the O-PS of *Brucella* smooth LPS is defined to contain A (for Abortus), M (for Melitensis) and C (for Common) epitopes [1,2]. *Brucella* O-PS is a linear homopolymer of 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (perosamine) subunits connected in α-1,2 linkage in A-dominant smooth *Brucella* strains, with every fifth residue connected in α-1,3 linkage in M-dominant smooth *Brucella* strains [3,4]. The O-PS is an immunodominant antigen, and infected animals usually develop robust antibodies to this antigen. Detection of anti-O-PS antibodies in the body fluids is the basis for several brucellosis diagnostic assays. At least in some animal species, anti-O-PS antibodies play a role in conferring enhanced protection against infections by *B. abortus*, *B. suis* and *B. melitensis* [5-7]. The O-PS also acts as a virulence factor by protecting the bacteria against complement-mediated lysis and the intracellular bactericidal milieu of phagocytic cells [8]. Consequently, the smooth *Brucella* strains are generally more virulent than their rough counterparts, which are typically attenuated [9]. *B. abortus* RB51, a laboratory derived stable rough attenuated strain, is used as a licensed live vaccine in the control of bovine brucellosis in the US and several other countries. Strain RB51 does not produce detectable levels of O-PS, and animals vaccinated with this strain do not develop anti-O-PS antibodies [10]. However, presence of low levels of M-like O-PS was detected in this strain [11].

The complete biosynthetic pathway of *Brucella* smooth LPS is yet to be determined. However, several genes are known to be essential for the biosynthesis of the O-PS [12-14]. These genes are located in two loci, *wbo* and *wbk*, on the *Brucella* chromosome [13]. Genes encoding four putative glycosyltransferases, *wboA*, *wboB*, *wbkA*, and *wbkE*, were identified to be involved in the polymerization of perosamine subunits leading to O-PS production [12-14]. The precise role of these four enzymes in O-PS synthesis is not yet established. In *B. abortus* RB51, the *wboA* gene is disrupted by an IS*711* element [15]. Sequence analysis of the *wbo* and *wbk* loci of strain RB51 did not reveal any other gene-disrupting mutations [16]. Complementation of strain RB51 with a functional *wboA* gene (RB51WboA) did not restore smooth phenotype, but resulted in the production of low levels of O-PS which remained in the cytoplasm [17]. In mouse models, strain RB51WboA vaccination induced low titers of anti-O-PS antibodies and conferred superior protection against virulent *B. abortus* and *B. melitensis* challenge [17,18].

The initial objective of this study was to determine if overexpression of *wbkA* or *wbkE* in strain RB51 would lead to production of detectable levels of O-PS. We cloned each gene in a multi-copy plasmid under a strong synthetic promoter and used the resulting plasmids to transform strain RB51. Although we did not detect any O-PS production by the recombinant strains, unexpectedly, the overexpression of *wbkA*, but not *wbkE*, in strain RB51 led to the development of hypermucoid colonies and the production of exopolysaccharide(s) (EPS) containing mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine. The EPS producing strain was similar to strain RB51 in its attenuation and vaccine efficacy characteristics.

Materials and methods

Bacterial strains

B. abortus strains RB51 and 2308 were from our culture collection. *B. neotomae and Pseudomonas* were obtained from American Type Culture Collection. Generation of strain

RB51WboA was described previously [17]. *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA) was used for the preparation of the necessary plasmid constructs. All bacteria were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) at 37°C. Ampicillin at 100 µg/ml was used for growing bacteria harboring plasmids. All experiments with virulent *Brucella* were performed in a BSL-3 facility approved for the select agents work.

Generation of recombinant strains RB51WbkA and RB51WbkE

The *wbkA* and *wbkE* genes were amplified by PCR using custom-designed primer-pairs and the genomic DNA of *B. abortus* 2308 as template. For the *wbkA* gene, the forward primer (5'-TT*TTCCATGG*CTCCCTACGAATACATTTGCA-3') and the reverse primer (5'-TTTT*TCTAGA*TTAATAGGTCATGAGCTTAGATTC-3') contained *Nco* I and *Xba* I restriction sites, respectively, at the 5' ends. Similarly, for the *wbkE* gene, the forward primer (5'-*AAGCTT*ATGCCGCATCTGTATTGGAGA-3') and the reverse primer (5'-*GGATCC*TCACTGCATCAGCGACGTATA-3') contained *Hind* III and *Bam* HI restriction sites, respectively, at the 5' ends. The amplified fragments were first cloned in pGEM-T Easy plasmid (Promega, Madison, WI) and sequenced to confirm the integrity of their nucleotide sequences. The inserts were subsequently excised from the pGEM-T plasmids using the restriction enzymes specific to the respective restriction sites engineered into the primers and cloned in the same sites of pBB4Trc plasmid. The resulting plasmids, pBB4TrcWbkA and pBB4TrcWbkE, were electroporated into strain RB51 as per previously described procedure [19] to generate strains RB51WbkA and RB51WbkE, respectively.

Sample preparation for electron microscopy

Freshly grown cultures of strains RB51 and RB51WbkA were mixed with an equal volume of stock buffer (0.1 M cacodylate buffer, pH 6.8) and incubated at room temperature for 10 mins. The bacterial cells were then pelleted by centrifugation and resuspended in

primary fixative solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M stock buffer, pH 6.8). After 1 hr of incubation, the cells were washed 2 times by centrifugation with the stock buffer, followed by a final washing with water. Secondary fixation of the bacteria was performed for 1 hr in a solution containing 1% osmium oxide and 1.5% potassium ferricyanide.

Scanning electron microscopy

After the secondary fixation, the bacterial cells were washed 2 times with water and filtered using a nucleopore membrane (25mm diameter, 0.2 mm pore size, Corning corp., 45 Nagog Park, Acton, MA). The bacterial cells were dehydrated using increasing concentrations of ethanol and the samples were mounted using a double-side carbon tape and sputter coated with platinum (Pt) for 60 secs prior to imaging. Images were obtained using FEI NOVA nanoSEM (FEI Company, Portland OR) with 5kV accelerating voltage.

Transmission electron microscopy

After the secondary fixation, the bacterial cells were washed 2 times with water and pelleted by centrifugation. Melted (45°C) agarose (1.5% w/v) was added to the tube and the bacterial cells were gently dispersed while keeping the tube in warm water. The dispersed bacterial cells and agarose mixture was cooled and extracted from the tube using 10% ethanol. Samples were then sliced and dehydrated using increasing concentrations of ethanol. Propylene oxide (PO) was used for a final rinse. Infiltration was carried out with 1/3 Spurr's resin (3 parts PO : 1 part resin) overnight, followed by further infiltration with 1/1 Spurr's resin (1 part PO : 1 part resin), 3/1 Spurr's resin (1 part PO : 3 parts resin) overnight, and finally with 100% Spurr's resin for 6 hrs in a rotator. The cells were then embedded in a fresh Spurr's resin and polymerized for 2 days at 60^{o}C. Samples were viewed under the FEI/Philips CM-10 transmission electron microscope (FEI Company, Hillsboro, OR) using an accelerating voltage of 80 kV.

SDS-PAGE and Western blotting

To detect O-PS expression and compare the protein profiles of RB51 and its recombinant strains, SDS-PAGE and Western blot analyses were performed as previously described [20]. As controls, antigen extracts of the strains RB51, RB51WboA and *B. neotomae* were used. Briefly, antigen extracts of strains RB51, RB51WboA, RB51WbkA, RB51WbkE and *B. neotomae* were separated on a 12.5% denaturing polyacrylamide gel by electrophoresis and stained with Coomassie Brilliant Blue. For Western blotting, the separated antigens were transferred onto a nitrocellulose membrane which was subsequently blocked with 5% skim milk and reacted with an appropriately diluted rat monoclonal antibody specific to *Brucella* O-PS [10]. The bound primary antibody was detected by reacting with horseradish peroxidase labelled-secondary antibody (KPL, Gaithersbur, MD), and developing the enzyme reaction using a colorimetric substrate (TMB substrate, KPL, Gaithersburg, MD).

Exopolysaccharide (EPS) staining

Recombinant strain RB51WbkA and the strain RB51 were grown for 24 hrs at 37°C in TSB with ampicillin and TSB alone, respectively. The bacteria were fixed with 4% paraformaldehyde (PFA) for 20 mins and used for staining.

Calcofluor white staining

For detection of polysaccharides, the fixed cells were washed three times with phosphate-buffered saline (PBS) (pH 8.5) and resuspended in 100 µl of the same buffer. 10 µl of the cell preparation was placed on a slide and one drop of calcofluor white stain (Fluorescent whitener 28, Sigma) was added to the cells. A coverslip was placed over the sample and the cells were visualized immediately using a Nikon A1R confocal laser scanning microscope with a 60X 1.4 NA oil immersion objective.

Lectin staining of EPS

Fluorescently labelled lectins (Vector laboratories Inc, Burlingame, California), conjugated with tetramethylrhodamine isothiocyanate (TRITC), with different sugar specificities (Table 4.1) were used to characterize the EPS composition. Fixed RB51 and RB51WbkA bacterial cells were stained with TRITC-labelled lectins (20 µg/ml in PBS). 4',6-diamidino-2-phenylindole (DAPI) was used to counterstain the bacterial cells. After an incubation for 30 mins in the dark at room temperature, the cells were washed three times with PBS, resuspended in 100 µl of the same buffer, and examined immediately using a Nikon A1R confocal laser scanning microscope with a 60X 1.4 NA oil immersion objective.

Competitive inhibition assay

The sugars D-(+)-galactose, D-(+)-mannose, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine (all from Sigma) were used to evaluate the carbohydrate binding specificities of the selected lectins (see Table 4.1). The sugars at a final concentration of 3 mg/ml or 100 mg/ml were mixed with solutions containing specific lectins at a concentration of 20 µg/ml. The mixtures were incubated for 30 mins in dark at room temperature to allow the sugars to bind with specific lectins. Each sugar plus lectin mixture was then used for staining the bacterial cells as described above.

Microtiter plate attachment assay

The attachment assay was performed as previously described [21], with few changes. Briefly, strain RB51WbkA was freshly grown overnight in 10 ml of TSB with antibiotic at 37°C. As controls, strain RB51 and *Pseudomonas* were grown overnight in 10 ml of TSB at 37°C. 100 µl of the overnight cultures was transferred to 10 ml of TSB, mixed thoroughly by vortexing, and a 200 µl of each resuspended culture was transferred to 8 wells in a 96-well polystyrene plate (USA Scientific, Ocala, FL). The plates were incubated at 37°C for

20 hrs. Then, the liquid medium was removed and the attached cells were washed with sterile PBS (pH 7.4). Plates were air dried for 45 mins and each well was stained with 150 µl of 1% crystal violet solution (GRAM'S solution, Merck) in water for 45 mins. The wells were then rinsed with water, air dried, and the bound stain was released by adding 200 µl of 95% ethanol. 100 µl from each well was transferred to a new microtiter plate and the intensity of the color was determined by reading the absorbance at 595 nm in a spectrophotometer (Molecular devices, Sunnyvale, CA).

Survival of strain RB51WbkA in mice

Female BALB/c mice of 4 to 6 weeks of age were used. Groups of 9 mice were inoculated with 2×10^8 CFU-equivalent of strains RB51 or RB51WbkA. At days 1, 7 and 21 post inoculation (p.i.), 3 mice from each group were euthanized by CO_2 asphyxiation followed by cervical dislocation. The spleens were collected aseptically and the *Brucella* CFUs per spleen were determined as previously described [10]. Briefly, the spleens were homogenized in TSB and ten-fold serial dilutions of the homogenates were plated on TSA plates for RB51 and TSA plates containing ampicillin for RB51WbkA. The bacterial CFUs were enumerated.

Mice immunizations

Groups of 4 female BALB/c mice of 4 to 6 weeks of age were used for the study. Mice were purchased from a commercial source (Harlan Laboratories, USA), and housed in cages with microisolator tops at 4 mice per cage. Feed and water were provided ad libitum. Housing conditions included standard 12 hrs light/dark cycle, controlled humidity (55%) and room temperature (22^oC). After 1 week of acclimatization, mice were administered with vaccine or control formulation.

Mice were immunized by intraperitoneal (i.p.) inoculation at day 0 with 2×10^8 CFU-equivalent of RB51 or RB51WbkA. Mice inoculated with saline served as control. Blood was collected from the mice by puncturing the retro-orbital plexus under anesthesia at 3

weeks and 6 weeks p.i. The serum was separated from the clotted blood and stored at -20°C until further use for the detection of antigen-specific antibodies by indirect enzyme-linked immunosorbent assay (ELISA).

Indirect ELISA

Indirect ELISA was used to determine the levels of serum immunoglobulin G (IgG), as well as IgG1, IgG2a, IgG2b and IgG3 isotypes with specificity to whole antigens of RB51 and RB51WbkA. Prior to coating the plates for ELISA, RB51 and RB51WbkA were heat-killed by incubating at 65°C for 1 hr. The antigens were diluted in carbonate buffer, pH 9.6, to a final concentration of 1×10^8 CFU-equivalent/ml. The wells of polystyrene plates (Nunc-Immunoplate with maxisorp surface) were coated with the diluted antigens (100 µl/well). Following overnight incubation at 4°C, the plates were washed four times in wash buffer (TBS at pH 7.4, 0.05% Tween 20) and blocked with 5% skim milk in TBS. After 1 hr of incubation at 37°C, mouse sera at 1 in 100 dilution in blocking buffer were added to the wells (50 µl/well). Each serum sample was tested in duplicate wells. Following incubation at room temperature for 4 hrs, the plates were washed four times in wash buffer and appropriately diluted horseradish peroxidase-labeled anti-mouse isotype specific conjugates (Southern Biotechnology Associates Inc, Birmingham, Alabama) were added to the wells (50 µl/well). After further 1 hr incubation at room temperature, the plates were washed four times, and 100 µl of substrate solution (TMB Microwell peroxidase substrate; KPL, Gaithersburg, MD) was added to each well. After 20 mins, the enzyme reaction was stopped by adding 100 µl of stop solution (0.185 M sulfuric acid) and the absorbance at 450 nm was recorded using microplate reader (Molecular devices, Sunnyvale, CA).

Protection experiment

Protection experiments were performed at Virginia Tech in an ABSL-3 facility that was approved for work with select agents. Female BALB/c mice of 4-6 weeks of age (Harlan Laboratories, USA) were used for the studies. Mice were housed in individually ventilated

cages with high-efficiency particulate arresting-filtered air. Feed and water were provided ad libitum. Housing conditions included standard 12 hrs light/dark cycle, controlled humidity (55%) and room temperature (22^{o}C). After 1 week of acclimatization, mice were administered with vaccine or control formulation.

Groups of four mice were vaccinated by i.p. inoculation with 2×10^{9} CFU-equivalent of RB51 or RB51WbkA. A group of mice inoculated with saline alone served as a control. 7 weeks p.i, each mouse was challenged by i.p. inoculation with 3×10^{4} CFU-equivalent of *B. abortus* 2308. 2 weeks post-challenge, the mice were euthanized and the bacterial burden in their spleens was enumerated as previously described [10].

Statistical analyses

Absorbance values of ELISA were analyzed for differences among the groups by performing analysis of variance with post hoc Bonferroni and Tukey for pair-wise comparison using SPSS version 21.0 (SPSS Inc., an IBM company, USA). For protection study, Student *t*-test modified for unequal variances between groups was performed to compare the log transformed bacterial loads in spleens of mice from each vaccinated group with the respective saline group. *P* values of <0.01 were considered significant.

Ethics statement

The protocols of the mice experiments performed in this study were approved by the Institutional Animal Care and Use Committees at Purdue University (Approval # 1112000488) and Virginia Tech (Approval # CVM-10-048). The animal studies were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Blood was collected from the retro-orbital plexus from mice under anesthesia. For anesthetizing mice, regulated concentration of anesthetic mixture (oxygen and isoflurane) was administered via a commercially available rodent anesthesia machine (Vetamac, Inc., Rossville, Indiana). Following blood collection, a drop of proparacaine hydrochloride ophthalmic solution

(Bausch & Lomb, Tampa, Florida) was placed on the eye to reduce pain. Mice infected with virulent *B. abortus* 2308 do not develop clinical disease or exhibit any signs of suffering for the duration of the experiments conducted in this study. Therefore, no humane endpoints were utilized for the mice in this study.

Results

B. abortus RB51 overexpressing wbkA gene displays a clumping phenotype

The colonies of strain RB51WbkA exhibited an excessively mucoid phenotype on agar plates and when grown in liquid culture, the bacteria formed strings and clumps (Fig. 4.1B). Strains RB51 (Fig. 4.1A) and RB51WbkE (data not shown), on the other hand, displayed uniform dispersion in liquid culture. As a result of the strikingly distinctive phenotype of strain RB51WbkA, several colonies were analyzed by RB51-specific PCR [15] and all of them were confirmed to be derived from strain RB51 (data not shown). One colony was selected for further studies.

Scanning and transmission electron microscopy were performed to examine the morphology of the bacteria. Strain RB51 culture contained well-defined coccobacilli, with relatively little or no visible extracellular material (Fig. 4.2A). In contrast, the recombinant strain RB51WbkA displayed formation of aggregates (Fig. 4.2B, left panel) containing bacterial cells and extraneous matrix material (Fig. 4.2B, left panel) characteristic of EPS. The recombinant bacterial cells also displayed altered cell walls (Fig. 4.2B).

Overexpression of wbkA in the strain RB51 does not result in O-PS synthesis

The antigen extracts of strain RB51WbkA did not react with Bru-38, a *Brucella* O-PS-specific monoclonal antibody, indicating the absence of O-PS expression (Fig. 4.3A, lane 3). As expected, strain RB51WboA and *B. neotomae* reacted with the antibody (Fig. 4.3A, lanes 5 and 6, respectively), while no reaction was detected with strain RB51 (Fig. 4.3A,

lane 2). Strain RB51WbkE also did not react with the O-PS specific monoclonal antibody (Fig. 4.3A, lane 4).

SDS-PAGE analysis did not reveal any apparent qualitative differences in the protein profiles between strains RB51 and RB51WbkA (Fig. 4.3B).

RB51WbkA bacterial clumps contain EPS

Bacterial extracellular matrices are frequently composed of polysaccharides. Therefore, a general EPS dye, calcofluor white, was used to determine the presence of EPS in the aggregate-forming strain RB51WbkA. As shown in Fig. 4.4B, a bright fluorescence was exhibited by strain RB51WbkA, indicating the presence of an extracellular polysaccharide composed of (1-4)- and/or (1-3)-β-D-linked glucan residues. The dye failed to bind with strain RB51 (Fig. 4.4A). The bacterial cells of strain RB51WbkA also bound with mannose-specific-TRITC-labelled LCA (Fig. 4.5B), mannose-specific-TRITC-labelled PSA (data not shown), galactose-specific-TRITC-labelled GSL I (data not shown), N-acetylglucosamine-specific-TRITC-labelled succinylated WGA (data not shown) and N-acetylgalactosamine-specific-TRITC-labelled SJA (Fig. 4.6B). However, strain RB51WbkA did not demonstrate any apparent binding to the lectins PHA-E and PHA-L (data not shown), which have specificity for complex polysaccharide structures. The bacterial cells of strain RB51 exhibited very weak fluorescence with mannose-specific-TRITC-labelled LCA (Fig. 4.5A) and PSA lectins (data not shown) when compared to strain RB51WbkA. Also, strain RB51 did not show any apparent binding with any of the other lectins tested, including TRITC-labelled SJA (Fig. 4.6A).

EPS produced by RB51WbkA contains mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine

In order to verify the specific lectin-binding pattern of strain RB51WbkA, competitive inhibition assay was carried out using 5 selected lectins (TRITC conjugated LCA, PSA, WGA, SJA and GSL I). Carbohydrate inhibition of the binding of specific TRITC-labelled

lectins to the EPS components was accomplished by using their respective target primary sugars (Table 4.1). The inhibition of lectin binding was evaluated microscopically by comparing the binding characteristic as well as the fluorescence intensity of strain RB51WbkA in presence and absence of the target carbohydrate. The observed binding pattern of the TRITC-labelled LCA and SJA are shown (Fig. 4.5 and 4.6, respectively). Original binding pattern of the TRITC-labelled LCA and SJA to the EPS(s) of the strain RB51WbkA in the absence of target carbohydrate is shown in Fig. 4.5B and 4.6B, respectively. Binding of the TRITC-labelled lectins to the EPS(s) was completely abrogated when the lectins were incubated with their target sugars at a concentration of 100 mg/ml (Fig. 4.5D and 4.6D). At carbohydrate concentrations of 3 mg/ml, the binding of TRITC-labelled LCA (Fig. 4.5C), PSA (data not shown), WGA (data not shown) and GSL I (data not shown) to the bacterial strain RB51WbkA was greatly reduced when compared with the binding pattern in the absence of the target sugars (Fig. 4.5B). However, at 3 mg/ml, N-acetylgalactosamine only slightly inhibited the binding of TRITC-labelled SJA lectin to the EPS(s) produced by strain RB51WbkA (Fig. 4.6C).

RB51WbkA displays increased adhesion property

We assessed the ability of strain RB51WbkA to adhere to a 96-well polystyrene plate when compared to strain RB51. *Pseudomonas* spp. was used as a positive control for this adherence assay. Strain RB51WbkA displayed significantly increased adherence to polystyrene wells when compared to strain RB51 (Fig. 4.7).

Bacterial persistence in mice spleens

Bacterial persistence of strain RB51WbkA in mouse spleens was determined and compared with that of strain RB51. As shown in Fig. 4.8, similar numbers of bacteria were present in the spleens of mice at days 1, 7 and 21 after inoculation with strains RB51WbkA and RB51. This result suggests that overexpression of *wbkA* did not affect the clearance of the recombinant RB51 strain in mice.

Induction of antigen-specific antibody responses

Serum samples collected from the groups of mice immunized with strains RB51 and RB51WbkA were analyzed in comparison with the sera obtained from the saline-inoculated group of mice (Fig. 4.9 and 4.10). Analysis with IgG-specific conjugate revealed that significantly higher level of RB51-specific IgG was present at 6 weeks p.i. in mice vaccinated with RB51 and RB51WbkA when compared with saline-inoculated mice (Fig. 4.9). Moreover, mice vaccinated with RB51 and RB51WbkA developed significantly higher levels of IgG2a, IgG2b and IgG3 isotypes specific to RB51 at 3 and 6 weeks p.i. when compared with saline-inoculated controls (Fig. 4.9). However, only vaccination with strain RB51WbkA resulted in a significant increase in RB51-specific IgG1 antibody at 6 weeks p.i. (Fig. 4.9).

Significantly increased levels of RB51WbkA-specific IgG, IgG2a and IgG3 antibodies were detected in serum of mice vaccinated with strains RB51 and RB51WbkA at 3 and 6 weeks p.i. than in saline-inoculated mice (Fig. 4.10). Moreover, mice vaccinated with RB51WbkA developed significantly higher levels of RB51WbkA-specific IgG at 3 and 6 weeks p.i., as well as RB51WbkA-specific IgG2a at 6 weeks p.i., when compared with RB51 vaccinated group of mice (Fig. 4.10). Only vaccination of mice with RB51WbkA resulted in significantly increased levels of RB51WbkA-specific IgG1 at 3 and 6 weeks p.i. when compared with the saline-inoculated mice (Fig. 4.10). Assay with IgG2b-specific conjugate revealed that RB51WbkA-specific IgG2b antibody was present at significantly higher levels in RB51 and RB51WbkA vaccinated groups of mice at 6 weeks p.i. when compared to saline-inoculated controls. However, at 3 weeks p.i., only the mice vaccinated with strain RB51WbkA developed significantly higher levels of RB51WbkA-specific IgG2b antibody than the saline-inoculated group (Fig. 4.10).

Protection against challenge with virulent B abortus 2308

Mice vaccinated with RB51 and RB51WbkA had significantly reduced number of virulent *Brucella* in their spleens when compared with the saline-inoculated group of mice (Fig.

4.11). However, there was no statistical difference in the splenic bacterial loads between the two vaccinated groups of mice.

Discussion

In this study, we unexpectedly discovered that overexpression of *wbkA* in strain RB51 confers an extreme mucoid and clumping phenotype that is associated with the production of EPS. It is known that *wbkA*, which encodes a putative glycosyltransferase, is essential for the O-PS and smooth LPS synthesis in *B. abortus* and *B. melitensis* [14]. However, the present study is the first to demonstrate the role of this gene product in EPS production in a *Brucella* strain. Till date, production of EPS was reported in certain recombinant gene-knockout or gene-overexpression strains of *B. melitensis* [22-25]. *B. melitensis* strain deficient in transcriptional regulator VjbR, which is involved in quorum sensing, was shown to produce EPS-like substance [23]. Overexpression of AiiD, an enzyme that degrades quorum sensing molecule acylhomoserine lactone (AHL), also leads to production of EPS in *B. melitensis* [24]. Interestingly, overexpression of MucR, an orthologue of a *Sinorhizobium meliloti* transcriptional regulator of its succinoglycan EPS, in *B. melitensis* resulted in clumping phenotype which is associated with EPS production [22]. A study also showed that EPS production occurs in *virB* mutants of *B. melitensis* [25]. Interestingly, overexpression of WbkA in strain VTRA1, a *B. abortus wboA* mutant [26], also resulted in the development of mucoid colonies that formed strings in liquid culture (result not shown). However, overexpression of WbkA in *B. abortus* 2308 resulted in mucoid colonies on solid media but the bacteria did not form strings in liquid culture (result not shown), suggesting the presence of some regulatory mechanism in the O-PS and EPS synthetic pathways. Currently, it is not clearly known if *Brucella* produces EPS in response to any natural environmental conditions or during the course of infection in mammalian hosts. However, above mentioned studies document the genetic competence of *B. melitensis* to produce EPS. Evidence for clumping phenotype and EPS production in *B. melitensis* under certain hypertonic culture conditions [22], and in *B. abortus* under microaerobic conditions [27] was also reported.

Brucella belongs to the class *Alphaproteobacteria*; several members of the class *Alphaproteobacteria* are known to produce EPS(s) during their life-cycle [28,29]. For example, *Sinorhizobium meliloti* produces an EPS, succinoglycan, which is essential for the expression of its full virulence including formation and invasion of nodules [28,30]. Interestingly, a family of glycosyltransferases was shown to be required for the synthesis of succinoglycan by *Sinorhizobium* spp [31]. Similarly, *Agrobacterium* strains also produce an EPS which is structurally identical to the succinoglycan EPS; however, it has been found to be dispensable for the formation of crown gall tumors by *Agrobacterium tumefaciens* [30]. The EPS produced by RB51WbkA increased the bacterial adherence to plastic matrix, but it did not have an effect on the bacteria's susceptibility to polymyxin B killing and persistence in mice. This suggests that the EPS did not prevent the rough LPS in the cell wall of RB51WbkA from external chemicals nor it was able to alter the attenuation characteristic of the bacteria.

The structure of EPS produced by RB51WbkA still needs to be determined. However, the qualitative results of our lectin binding studies indicate the presence of mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine in the EPS. This EPS composition is similar to that reported for *B. melitensis* [24], where mannose was detected to be the primary sugar in the EPS. Several of our attempts to purify the EPS from RB51WbkA were unsuccessful. This could have been because of the molecular nature of the complexes between EPS and rough LPS of RB51. Any future studies should take this into consideration for developing strategies for purification of EPS from RB51WbkA.

Two-component systems have been found to regulate the EPS formation in many plant pathogenic bacteria, including *Sinorhizobium meliloti* [32,33]. These systems can sense the specific bacterial requirements during pathogenesis and can subsequently regulate the production of EPS. Global regulatory mechanisms, such as the two-component systems, quorum sensing (QS), also fine-tune the synthesis and secretion of EPS, leading to an increase in synthesis during nutritional stress and a decrease in EPS synthesis during growth of cells under nutrient sufficient environment. A previous study has demonstrated the formation of clumps in liquid culture by a *vjbR* mutant strain of *B. melitensis* [23]. Mutation in *vjbR* render the bacteria incapable of responding to the presence of AHL. One

explanation of this phenomenon is that the bacteria are unable to regulate the *vjbR*-dependent AHL-mediated repression of genes which are involved in clumping [23]. VjbR was found to regulate the EPS synthesis and/or export, and also the production of several outer membrane proteins (OMPs) [23]. Another study documented the induction of a similar clumping phenotype by *Brucella* overexpressing AHL-acylase *aiiD* [24]. The authors hypothesize that the overexpression of *aiiD* leads to the degradation of all of the intrinsically synthesized AHLs, resulting in unbound VjbR regulators which activate the expression of genes involved in clumping [24]. *wbkA* was found to be a QS-target using proteomic and microarray analysis [34]. It is interesting that WbkA is the only glycosyltransferase identified as a target of QS-regulators in the study. Further studies need to be undertaken to investigate the role of the QS-regulators in modulating the expression of *wbkA* under specific environmental conditions and the effect of gene-interplay on the level of the EPS production.

Mice immunized with strain RB51WbkA appeared to develop antibodies to the EPS, as significantly higher levels of IgG antibodies were detected to be specific to RB51WbkA than RB51. IgG1 and IgG2a were the prominent isotypes that contributed to this difference. Nevertheless, both RB51 and RB51WbkA vaccines induced the same level of protective response against the virulent *B. abortus* challenge, suggesting the minimal, if any, role of EPS in modulating immune responses in mice.

In conclusion, our studies demonstrate that the overexpression of *wbkA* in RB51 results in the production of EPS that confers increased adherence property to the polystyrene surfaces. This EPS was found to contain mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine. This finding adds to the growing evidence for the EPS synthesis in *Brucella*. EPS has previously been shown to aid some bacteria to survive in a hostile environment, evade the immune mechanism of the host, and adhere to the host cells [35,36]. Further studies to identify the role of EPS in affecting the bacterial fitness under different environmental conditions would help delineate the precise contribution of EPS to the pathogenesis of *Brucella*.

References

[1] Diaz, R., Jones, L. M., Leong, D., Wilson, J. B., 1968. Surface antigens of smooth brucellae. J Bacteriol 96, 893-901.

[2] Hurvell, B., Lindberg, A. A., 1973. Serological cross reactions between different *Brucella* species and *Yersinia enterocolitica*. Acta Pathol Microbiol Scand Sect B 81, 113-119.

[3] Bundle, D. R., Cherwonogrodzky, J. W., Perry, M. B., 1987. The structure of the lipopolysaccharide O-chain (M antigen) and polysaccharide B produced by *Brucella melitensis* 16 M. FEBS lett 216, 261-264.

[4] Caroff, M., Bundle, D. R., Perry, M. B., Cherwonogrodzky, J. W., Duncan, J. R., 1984. Antigenic S type lipopolysaccharide of *Brucella abortus* 1119-3. Infect Immun 46, 384-388.

[5] Vitry, M. A., Mambres, D. H., Trez, C. D., Akira, S., Ryffel, B., Letesson, J. J., Muraille, E., 2014. Humoral immunity and $CD4^+$ Th1 cells are both necessary for a fully protective immune response upon secondary infection with *Brucella melitensis*. J Immunol 192, 3740-52.

[6] Araya, L. N., Winter, A. J., 1990. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infect Immun 58, 254-256.

[7] Ficht, T. A., Kahl-Mcdonagh, M. M., Arenas-Gamboa, A. M., Rice-Ficht, A. C., 2009. Brucellosis: the case for live, attenuated vaccines. Vaccine 27, D40-D3.

[8] Lapaque, N., Moriyon, I., Moreno, E., Gorvel, J. P., 2005. *Brucella* lipopolysaccharide acts as a virulence factor. Curr Opin Microbiol 8, 60-66.

[9] Allen, C. A., Adams, G. L., Ficht, T. A., 1998. Transposon-derived *Brucella abortus* rough mutants are attenuated and exhibit reduced intracellular survival. Infect Immun 66, 1008-1016.

[10] Schurig, G. G., Roop II, R. M., Bagchi, T., Boyle, S., Buhrman, D., Sriranganathan, N., 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 28, 171-188.

[11] Cloeckaert, A., Zygmunt, M. S., Guilloteau, L. A., 2002. *Brucella abortus* vaccine strain RB51 produces low levels of M-like O-antigen. Vaccine 20, 1820-1822.

[12] Godfroid, F., Cloeckaert, A., Taminiau, B., Danese, I., Tibor, A., De Bolle, X., Mertens, P., Letesson, J. J., 2000. Genetic organization of the lipopolysaccharide O-antigen biosynthesis region of *Brucella melitensis* 16M (*wbk*). Res Microbiol 151, 655-668.

[13] Zygmunt, M. S., Blasco, J. M., Letesson, J. J., Cloeckaert, A., Moriyn, I., 2009. DNA polymorphism analysis of *Brucella* lipopolysaccharide genes reveals marked differences in O-polysaccharide biosynthetic genes between smooth and rough *Brucella* species and novel species-specific markers. BMC Microbiol 9, 92.

[14] Gonzales, D., Grillo, M. J., De Miguel, M. J., Ali, T., Arce-Gorvel, V., Delrue, R. M., Conde-Alvarez, R., Munoz, P., Lopez-Goni, I., Iriarte, M., Marin, C. M., Weintraub, A., Widmalm, G., Zygmunt, M., Letesson, J. J., Gorvel, J. P., Blasco, J. M., Moriyon, I., 2008. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLOS ONE 3, e2760.

[15] Vemulapalli, R., McQuiston, R., Schurig, G. G., Sriranganathan, N., Halling, S. M., Boyle, S. M., 1999. Identification of an *IS711* element interrupting the *wboA* gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. Clin Diagn Lab Immun 6, 760-764.

[16] Adone, R., Muscillo, M., La Rosa, G., Francia, M., Tarantino, M., 2011. Antigen, immunologic and genetic characterization of rough strains *B. abortus* RB51, *B. melitensis* B115 and *B. melitensis* B18. PLOS ONE 6, e24073.

[17] Vemulapalli, R., He, Y., Buccolo, L. S., Boyle, S. M., Sriranganathan, N., Schurig, G. G., 2000. Complementation of *Brucella abortus* RB51 with a functional *wboA* gene results in O-antigen synthesis and enhanced vaccine efficacy but no change in rough phenotype and attenuation. Infect Immun 68, 3927-3932.

[18] Vemulapalli, R., Contreras, A., Sanakkayala, N., Sriranganathan, N., Boyle, S. M., Schurig, G. G., 2004. Enhanced efficacy of recombinant *Brucella abortus* RB51 vaccines against *B. melitensis* infection in mice. Vet Microbiol 102, 237-245.

[19] McQuiston, J. R., Schurig, G. G., Sriranganathan, N., Boyle, S. M., 1995. Transformation of *Brucella* species with suicide and broad host-range plasmids. Methods Mol Biol 47, 143-148.

[20] Vemulapalli, R., Duncan, A. J., Boyle, S. M., Sriranganathan, N., Toth, T. E., Schurig, G. G., 1998. Cloning and sequencing of *yajC* and *secD* homologs of *Brucella abortus* and demonstration of immune responses to YajC in mice vaccinated with *B. abortus* RB51. Infect Immun 66, 5684-91.

[21] Djordjevic, D., Wiedmann, M., McLandsborough, L. A., 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol 68, 2950-2958.

[22] Mirabella, A., Terwagne, M., Zygmunt, M. S., Cloeckaert, A., De Bolle, X., Letesson, J. J., 2013. *Brucella melitensis* MucR, an orthologue of *Sinorhizobium meliloti* MucR, is involved in resistance to oxidative, detergent, and saline stresses and cell envelope modifications. J Bacteriol 195, 453-65.

[23] Uzureau, S., Godefroid, M., Deschamps, C., Lemaire, J., De Bolle, X., Letesson, J. J., 2007. Mutations of the quorum sensing-dependent regulator VjbR lead to drastic surface modifications in *Brucella melitensis.* J Bacteriol 189, 6035-6047.

[24] Godefroid, M., Svensson, M. V., Cambier, P., Uzureau, S., Mirabella, A., De Bolle, X., Cutsem, P. V., Widmalm, Letesson, J. J., 2010. *Brucella melitensis* 16M produces a mannan and other extracellular matrix components typical of a biofilm. FEMS Immunol Med Mic 59, 364-377.

[25] Wang, Y., Chen, Z., Qiao, F., Zhong, Z., Xu, J., Wang, Z., Du, X., Qu, A., Yuan, J., Jia, L., Song, H., Sun, Y., Huang, L., 2010. The type IV secretion system affects the expression of Omp25/Omp31 and the outer membrane properties of *Brucella melitensis*. FEMS Microbiol Lett 303, 92-100.

[26] Winter, A. J., Schurig, G. G., Boyle, S. M., Sriranganathan, N., Bevins, J. S., Enright, F. M., Elzer, P. H., Kopec, J. D., 1996. Protection of BALB/c mice against homologous and heterologous species of *Brucella* by rough strain vaccines derived from *Brucella melitensis* and *Brucella suis* biovar 4. Am J Vet Res 57, 677-683.

[27] Almiron, M. A., Roset, M. S., Sanjuan, N., 2013. The aggregation of *Brucella abortus* occurs under microaerobic conditions and promotes desiccation tolerance and biofilm formation. Open Microbiol J 7, 87-91.

[28] Leigh, J. A., Signer, E. R., Walker, G. C., 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. P Natl Acad Sci USA 82, 6231-6235.

[29] Stredansky, M., Conti, E., 1999. Succinoglycan production by solid-state fermentation with *Agrobacterium tumefaciens*. Appl Microbiol Biot 52, 332-337.

[30] Leigh, J. A., Coplin, D. L., 1992. Exopolysaccharides in plant-bacterial interactions. Annu Rev Microbiol 46, 307-346.

[31] Glucksmann, A. M., Reuber, L. T., Walker, G. C., 1993. Family of glycosyl transferases needed for the synthesis of succinoglycan by *Rhizobium meliloti*. J Bacteriol 175, 7033-7044.

[32] Cheng, H. P., Walker, G. C., 1998. Succinoglycan production by *Rhizobium meliloti* is regulated through the ExoS-ChvI two-component regulatory system. J Bacteriol 180, 20-26.

[33] Skorupska, A., Janczarek, M., Marczak, M., Mazur, A., Krol, J., 2006. Rhizobial exopolysaccharides: genetic control and symbiotic functions. Microb Cell Fact 5, 7.

[34] Uzureau, S., Lemaire, J., Delaive, E., Dieu, M., Gaigneaux, A., Raes, M., De Bolle, X., Letesson, J. J., 2010. Global analysis of quorum sensing targets in the intracellular pathogen *Brucella melitensis* 16M. J Proteome Res 9, 3200-3217.

[35] D'Haeze, W., Holsters, M., 2004. Surface polysaccharides enable bacteria to evade plant immunity. Trends Microbiol 12, 555-561.

[36] Zhu, M., Takenaka, S., Sato, M., Hoshino, E., 2001. Influence of starvation and biofilm formation on acid resistance of *Streptococcus mutans*. Oral Microbiol Immun 16, 24-27.

Table 4.1. Carbohydrate-binding specificities of lectins employed in this study for the staining of EPS(s) produced by the recombinant strain RB51WbkA.

Lectin	Abbreviation	Source	Primary sugar specificity
Griffonia simplicifolia lectin I	GSL I	*Griffonia simplicifolia (Bandeiraea)* seeds	Galactose
Lens culinaris lectin	LCA	*Lens culinaris* (lentil) seeds	Mannose
Phaseolus vulgaris Erythroagglutinin	PHA-E	*Phaseolus vulgaris* (Red Kidney Bean) seeds	Complex structures
Phaseolus vulgaris Leucoagglutinin	PHA-L	*Phaseolus vulgaris* (Red Kidney Bean) seeds	Complex structures
Pisum sativum agglutinin	PSA	*Pisum sativum* (Pea) seeds	Mannose
Wheat germ agglutinin, succinylated	Succinylated WGA	*Triticum vulgaris* (wheat germ)	N-acetylglucosamine
Sophora japonica agglutinin	SJA	*Sophora japonica* (Japanese Pagoda Tree) seeds	N-acetylgalactosamine

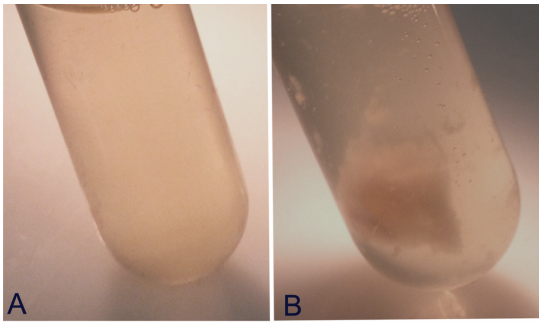

Figure 4.1. Observation of the clumping phenotype of the recombinant strain *B. abortus* RB51WbkA. (A) *B. abortus* RB51; (B) strain *B. abortus* RB51 overexpressing the *wbkA* gene.

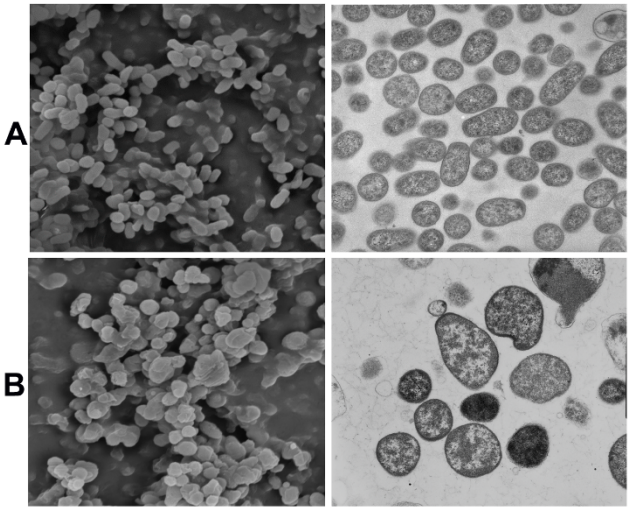

Figure 4.2. Electron microscopic images of strain RB51WbkA. Scanning electron micrographs (left panels; ×30,000) and transmission electron micrographs (right panels; ×15,000) of the strains (A) *B. abortus* RB51 and (B) *B. abortus* RB51WbkA. The recombinant strain RB51WbkA displays a clumping phenotype and produces EPS(s). Scanning electron micrograph show the clumping phenotype of the recombinant strain RB51WbkA (B, left panel).

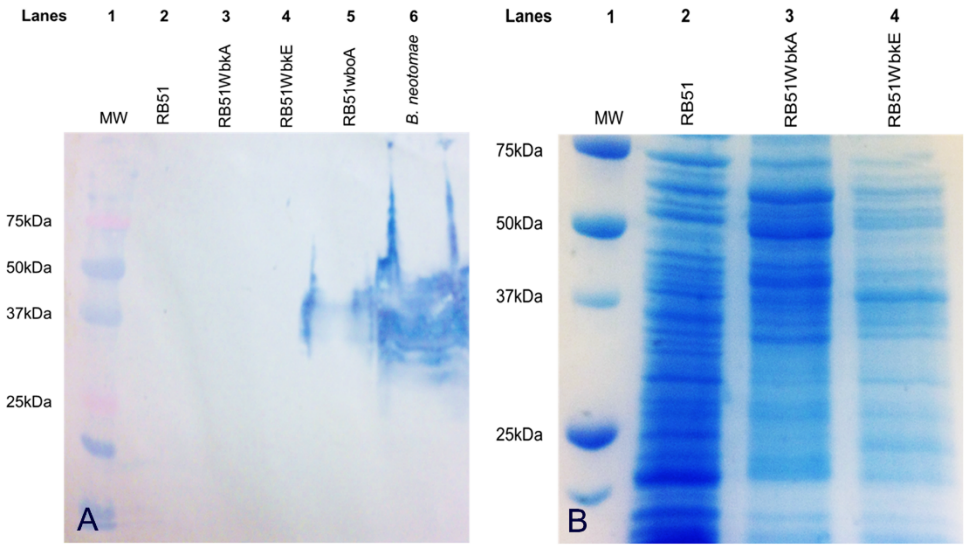

Figure 4.3. (A) Western blot analysis to detect O-PS expression by the strain RB51WbkA and (B) Coomassie Brilliant Blue staining of the polyacrylamide gel to detect difference in the protein profiles of strain RB51 and strain RB51WbkA. Whole antigens of the strain RB51, recombinant strain RB51WbkA, RB51WbkE, RB51WboA and *B. neotomae* were separated by 12.5% SDS-PAGE and analyzed by (A) Western blotting with O-PS specific monoclonal antibody, Bru-38. Lane 1: molecular weight marker (MW) in kilodaltons (kDa); lane 2: strain RB51; lane 3: recombinant strain RB51WbkA; lane 4: recombinant strain RB51WbkE; lane 5: recombinant strain RB51WboA; lane 6: strain *B. neotomae*. Whole antigens of the strain RB51, RB51WbkA and RB51WbkE were analyzed for any differences in the expressed protein profiles by (B) Coomassie Brilliant Blue staining of polyacrylamide gel.

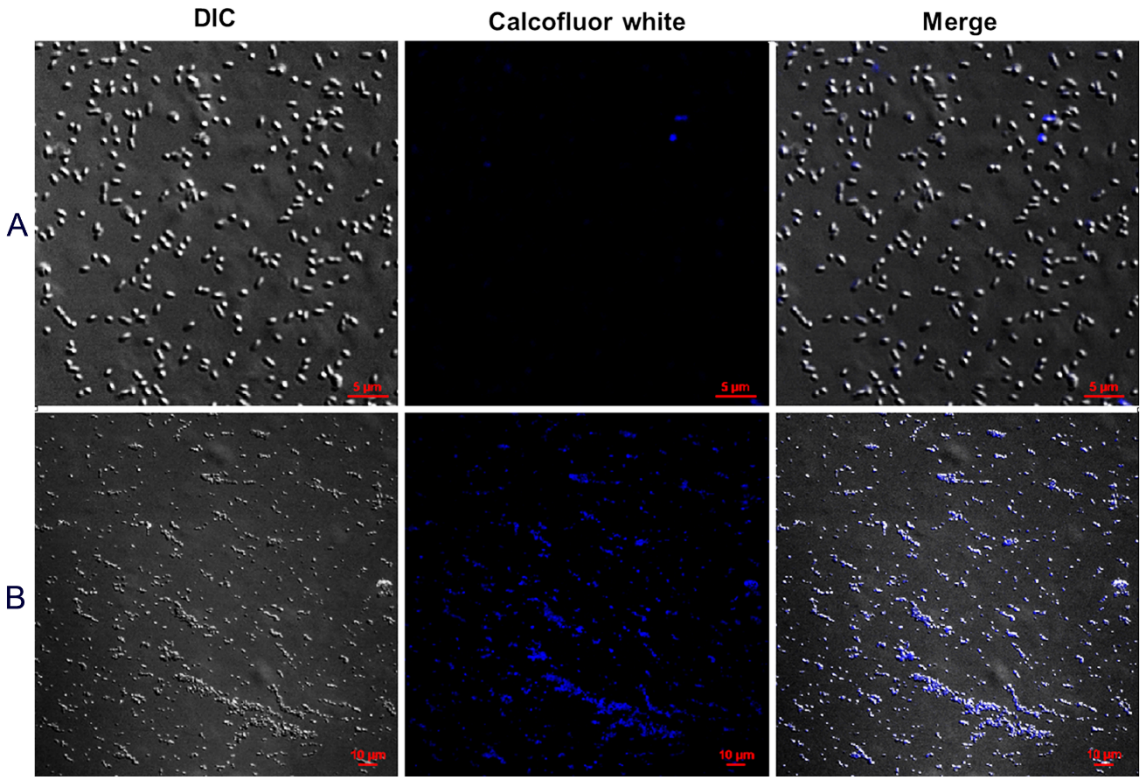

Figure 4.4. Confocal microscopy of the EPS(s) produced by the recombinant strain RB51WbkA. Interactions between the calcofluor white stain and the aggregates formed by the strain RB51WbkA were visualized using confocal laser scanning microscopy. (A) Strain RB51; (B) strain RB51WbkA. Selected differential interference contrast (DIC) images (left panel) and fluorescent images (middle panel) merged are shown (right panel). All images were acquired using the same settings and adjusted for display using the same brightness/contrast settings.

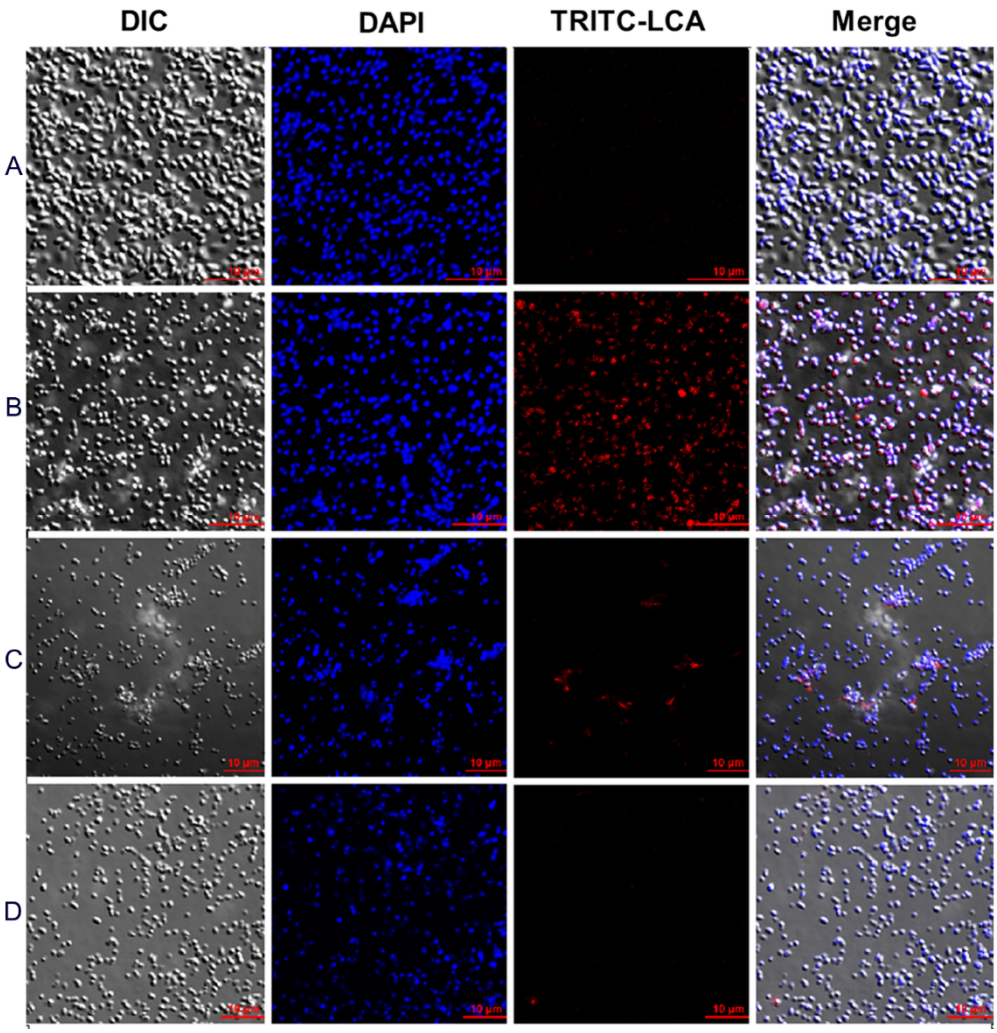

Figure 4.5. Confocal laser scanning microscopy showing the interactions between the fluorescently labelled LCA lectin and the (A) strain RB51; (B, C & D) strain RB51WbkA. Panel A and B show the bacterial cells stained with TRITC-labelled LCA lectin (red) in the absence of the target carbohydrate. Panel C & D show the bacterial cells of the strain RB51WbkA stained with TRITC-labelled LCA lectin preincubated with (C) 3mg/ml mannose and (D) 100 mg/ml mannose, prior to staining. DAPI (blue) was used to stain the bacterial nuclei. Selected differential interference contrast (DIC) images (left panel) and fluorescent images (middle panels) merged are shown (right panel). All images were acquired using the same settings and adjusted for display using the same brightness/contrast settings.

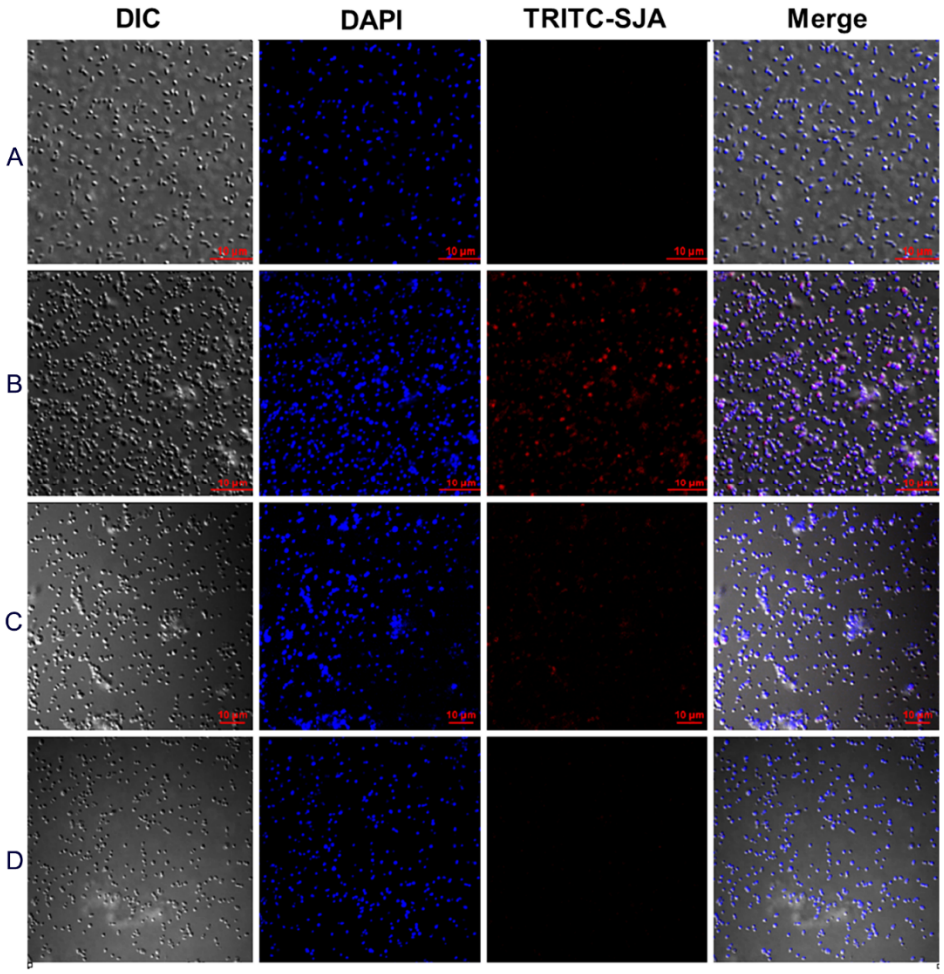

Figure 4.6. Confocal laser scanning microscopy showing the interactions between the fluorescently labelled SJA lectin and the (A) strain RB51; (B, C & D) strain RB51WbkA. Panel A and B show the bacterial cells stained with TRITC-labelled SJA lectin (red) in the absence of the target carbohydrate. Panel C & D show the bacterial cells of the strain RB51WbkA stained with TRITC-labelled SJA lectin preincubated with (C) 3mg/ml N-acetylgalactosamine and (D) 100 mg/ml N-acetylgalactosamine, prior to staining. DAPI (blue) was used to stain the bacterial nuclei. Selected differential interference contrast (DIC) images (left panel) and fluorescent images (middle panels) merged are shown (right panel). All images were acquired using the same settings and adjusted for display using the same brightness/contrast settings.

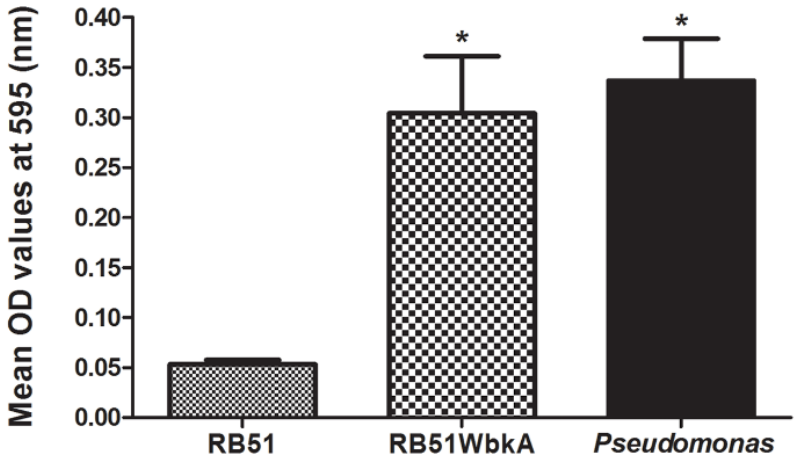

Figure 4.7. Quantification of the microtiter plate adhesion assay of the recombinant strain RB51WbkA. The surface attachment of the strain RB51, recombinant strain RB51WbkA and *Pseudomonas* spp. is shown. Quantification of the microtiter plate adhesion is done by staining the adherent bacterial cells with crystal violet, subsequent solubilization of the crystal violet stain in ethanol and spectrophotometric determination of absorbance at 595 nm. Results are shown as mean ± standard deviation of absorbance of eight independent samples. *Significantly different from the RB51 strain ($P < 0.01$). OD, optical density.

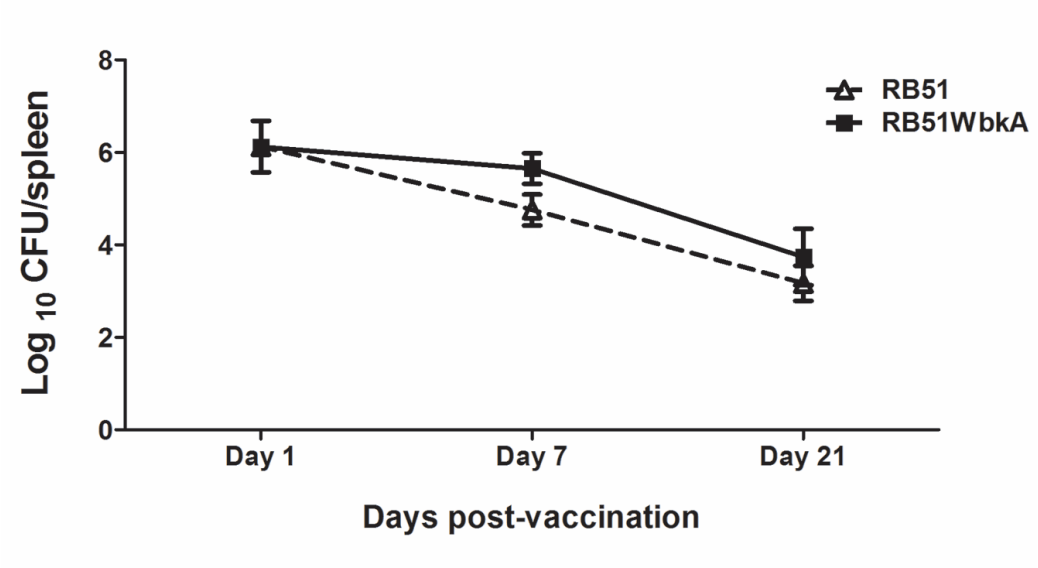

Figure 4.8. Determination of bacterial persistence in spleens of mice immunized with recombinant strain RB51WbkA. Mice spleens were collected at day 1, day 7 and day 21 after vaccination with live RB51 and live recombinant RB51WbkA. The *Brucella* CFUs in their spleens were determined. Results are shown as mean ± standard deviation (n=3) of the log CFU of *Brucella* recovered from spleens. Overexpression of *wbkA* in RB51 does not result in increased survival in mice.

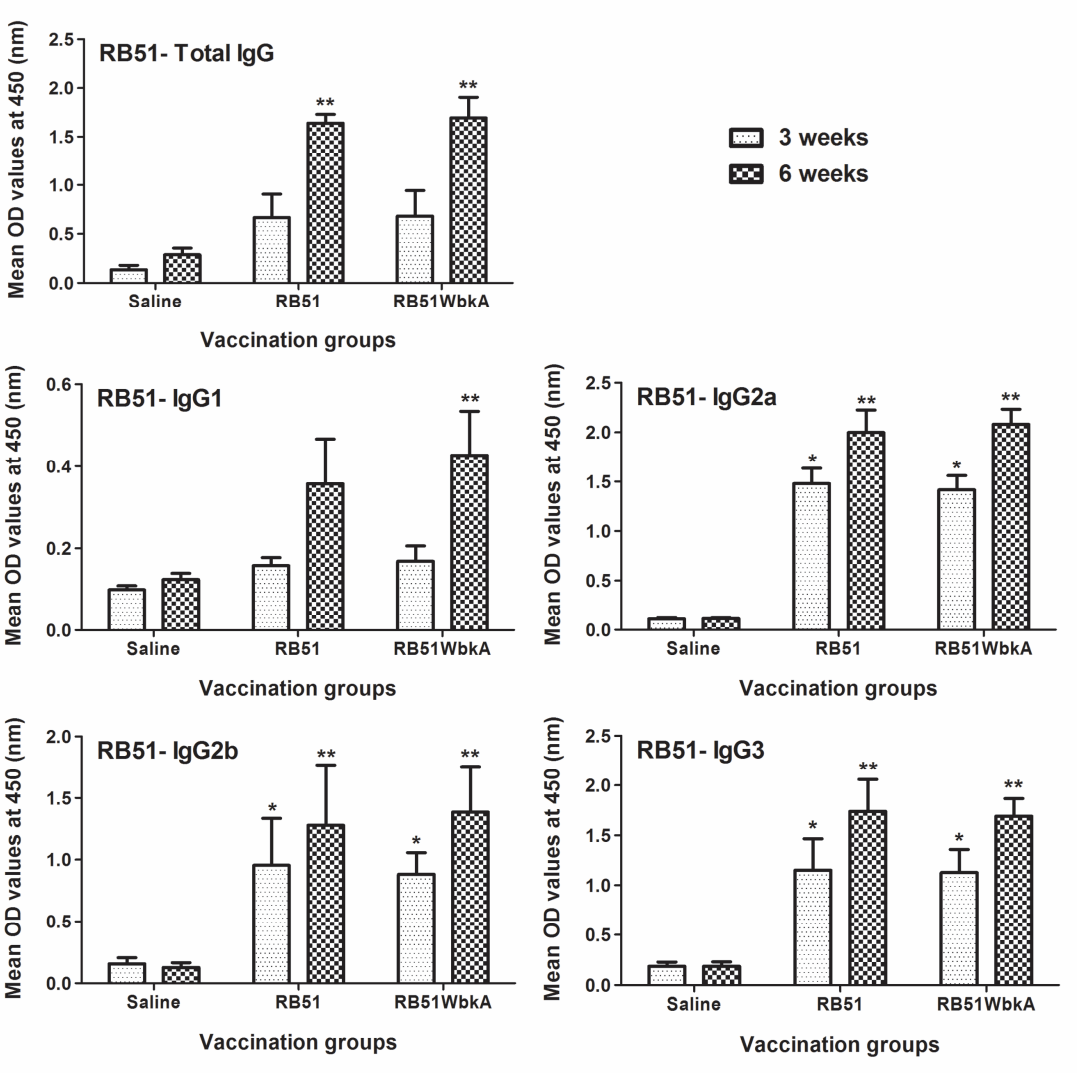

Figure 4.9. Detection of RB51-specific antibodies in serum of mice vaccinated with live recombinant RB51WbkA. Mice were vaccinated with live RB51, live recombinant RB51WbkA, or inoculated with saline. Serum samples were collected at 3 and 6 weeks after vaccination, and were diluted 1 in 100 and assayed for the presence of total antigen-specific IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies by indirect ELISA. Results are shown as mean ± standard deviation (n=4) of absorbance of the color developed. *Significantly different from the corresponding saline group at week 3 ($P < 0.01$). **Significantly different from the corresponding saline group at week 6 ($P < 0.01$). OD, optical density.

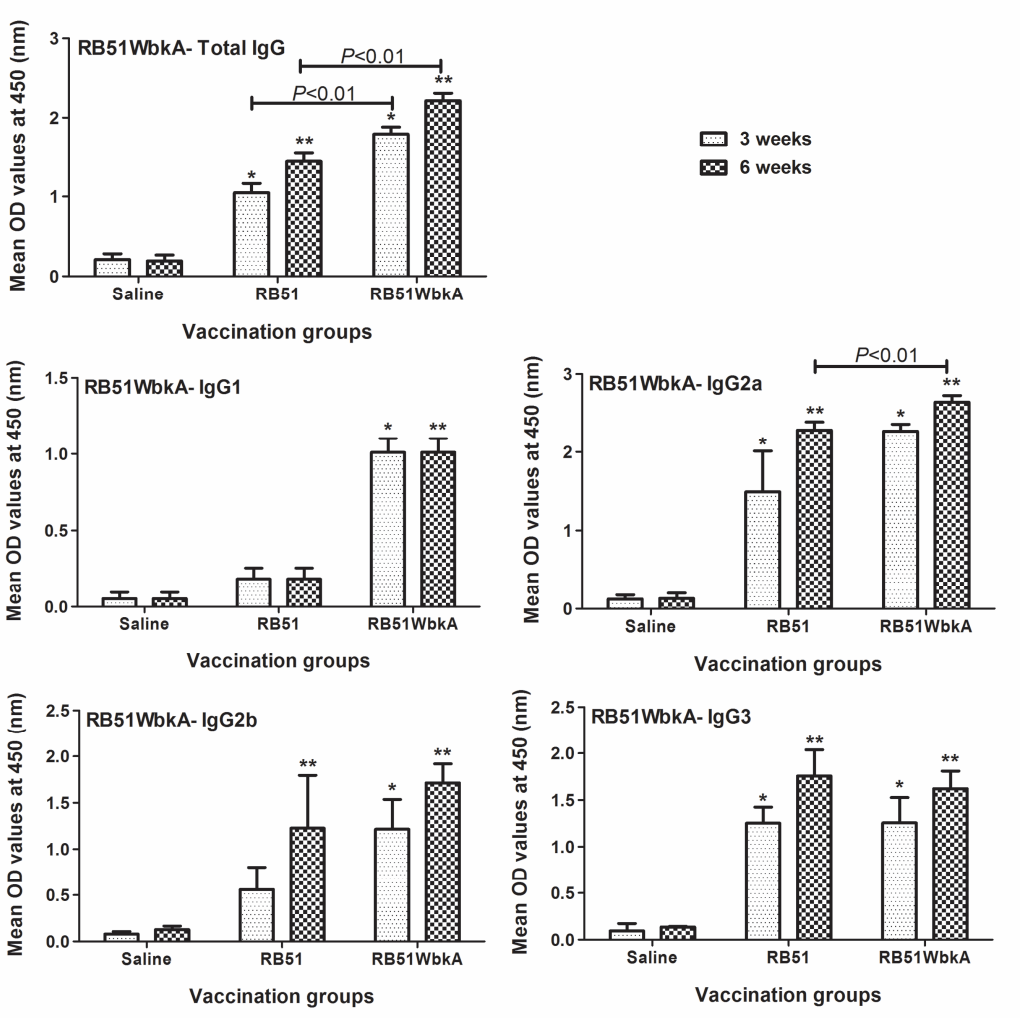

Figure 4.10. Detection of RB51WbkA-specific antibodies in serum of mice vaccinated with live recombinant RB51WbkA. Mice were vaccinated with live RB51, live recombinant RB51WbkA, or inoculated with saline. Serum samples were collected at 3 and 6 weeks after vaccination, and were diluted 1 in 100 and assayed for the presence of total antigen-specific IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies by indirect ELISA. Results are shown as mean ± standard deviation (n=4) of absorbance of the color developed. *Significantly different from the corresponding saline group at week 3 ($P < 0.01$). **Significantly different from the corresponding saline group at week 6 ($P < 0.01$). OD, optical density.

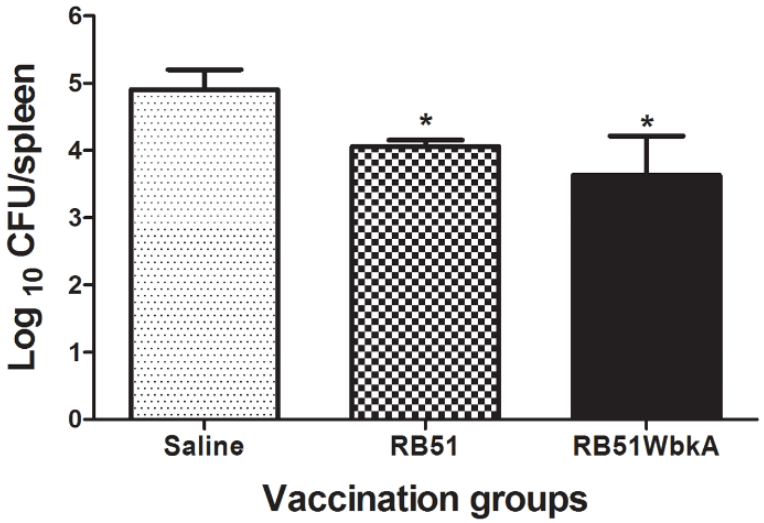

Figure 4.11. Detection of the protective efficacy of RB51WbkA against challenge with the virulent strain *B. abortus* 2308. Mice vaccinated with live RB51, live recombinant RB51WbkA and saline-inoculated mice were challenged by i.p. inoculation of 3×10^4 CFU-equivalent of virulent strain 2308. 2 weeks post-challenge, the mice were euthanized and the *Brucella* CFUs in their spleens were determined. Results are shown as mean ± standard deviation (n=5) of the log CFU of *Brucella* recovered from spleens. *Significantly different from the corresponding saline group ($P < 0.01$).

CHAPTER 5. OVEREXPRESSION OF *WBKF* GENE IN *BRUCELLA ABORTUS* RB51WBOA LEADS TO O-POLYSACCHARIDE EXPRESSION AND ENHANCED PROTECTION AGAINST *BRUCELLA ABORTUS* 2308 AND *BRUCELLA MELITENSIS* 16M IN A MURINE BRUCELLOSIS MODEL

Abstract

Brucella abortus strain RB51 is an attenuated, stable, rough mutant derived in the laboratory from the virulent strain *B. abortus* 2308. Animals vaccinated with strain RB51 do not produce antibodies to the O-polysaccharide (O-PS) of the smooth lipopolysaccharide (LPS). Previous studies have shown that the *wboA* gene, which encodes a glycosyltransferase required for the synthesis of the O-PS, is disrupted in strain RB51 by an IS*711* element. Complementation of strain RB51 with a functional *wboA* gene (strain RB51WboA) results in low levels of cytoplasmic O-PS synthesis, but it does not confer a smooth phenotype. In this study, we asked if increasing the amount of bactoprenol priming precursors in strain RB51WboA would result in smooth LPS synthesis. To achieve this, we overexpressed the *wbkF* gene, which encodes undecaprenyl-glycosyltransferase involved in bactoprenol priming for O-PS polymerization, in strain RB51WboA to generate strain RB51WboAKF. In comparison with strain RB51WboA, strain RB51WboAKF expressed higher levels of O-PS. Immuno-electron microscopy revealed that the expressed O-PS was present in the cell wall of RB51WboAKF. However, RB51WboAKF strain exhibited rough phenotypic characteristic in acriflavine agglutination test. Mice immunized with strain RB51WboAKF developed increased levels of smooth LPS-specific serum antibodies, primarily of IgG2a and IgG3 type, when

compared with those immunized with strain RB51WboA. Splenocytes from the RB51WboAKF vaccinated group of mice secreted higher levels of antigen-specific IFN-γ, IL-10 and TNF-α when compared to those of the RB51 or RB51WboA vaccinated groups. Also, increased numbers of antigen-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T lymphocytes were detected in RB51WboAKF vaccinated mice. Immunization of mice with strain RB51WboAKF conferred enhanced protection against virulent *B. abortus* 2308 and *B. melitensis* 16M when compared with the RB51 and RB51WboA immunized groups of mice. These results suggest that, in addition to *wboA* gene, mutations affecting expression of several other genes involved in the synthesis of O-PS and smooth LPS contribute to the rough phenotype of strain RB51. Our results also suggest that strain RB51WboAKF could be a more efficacious vaccine than its parent strain in natural hosts.

Introduction

Bacteria belonging to the genus *Brucella* are Gram-negative, facultative intracellular coccobacilli that cause one of the most frequently encountered zoonosis worldwide known as brucellosis. *Brucella* spp. infect a wide variety of mammals and can cause abortions in natural hosts and undulant fever in humans. They can exhibit smooth or rough colony morphology depending on the structure of their lipopolysaccharide (LPS). Except for *B. canis* and *B. ovis*, all other *Brucella* species known to date possess a surface-exposed O-polysaccharide (O-PS) in their LPS moiety and exhibit a smooth phenotype. Rough strains are devoid of the O-PS in their LPS and are generally attenuated than their smooth counterparts [1-5]. O-PS is a linear homopolymer of 4,6-dideoxy-4-formamido-α-D-mannopyranosyl (perosamine) subunits. These subunits are connected in α-1,2 linkage in A-dominant strains, with every fifth residue connected in α-1,3 linkage in M-dominant *Brucella* strains [6,7]. O-PS protects the smooth *Brucella* spp. from host defense mechanisms including complement-mediated lysis and the phagocytic microbicidal functions [1,8]. Acquired immunity against brucellosis is primarily cell-mediated and IFN-γ producing $CD4^+$ and $CD8^+$ T cells play a major role in protection against *Brucella* [9]. However, O-PS-specific antibodies also participate in enhancing the protection against

virulent *B. abortus* 2308, *B. melitensis* and *B. suis* [10-13]. Currently, *B. abortus* strain RB51 is used as a licensed cattle vaccine in United States and several other countries [4]. It is a laboratory-derived rough, stable mutant of virulent *B. abortus* 2308 [4]. It can express low levels of M-like O-PS; however, it does not induce detectable levels of antibodies to the O-PS in vaccinated animals [4,14].

The complete smooth LPS biosynthetic pathway is still not fully understood. The genes that are essential for the O-PS biosynthesis are located on two genetic loci, *wboA* and *wbkA*, of *Brucella* genome [15]. Previous studies have demonstrated that an *IS711* element disrupts the *wboA* gene, which encodes a glycosyltransferase required for the synthesis of O-PS in *Brucella*, in strain RB51 [16]. Complementing strain RB51 with a functional *wboA* gene (strain RB51WboA) resulted in the expression of low levels of intra-cytoplasmic O-PS; however, it did not restore the smooth phenotype [17]. Interestingly, when compared with strain RB51, immunization of mice with RB51WboA resulted in superior level of protection against virulent *B. abortus* 2308 and *B. melitensis* 16M strain [17,18]. Recent nucleotide sequence analysis of genes in the *wbo* and *wbk* loci did not reveal any other apparent mutation that could affect the O-PS synthesis in strain RB51 [19].

The objective of this study was to determine if increasing the amount of bactoprenol priming precursors in strain RB51WboA would result in smooth LPS synthesis and enhanced protection against virulent *Brucella* challenge. Gene *wbkF*, encoding putative undecaprenyl-glycosyltransferase, was identified to be essential for O-PS synthesis in *Brucella* [15]. We cloned the *wbkF* gene under a strong promoter in a multi-copy plasmid vector harboring the *wboA* gene and transformed strain RB51 with the resulting plasmid pBBR/wboAKF to generate strain RB51WboAKF. Our results indicate that strain RB51WboAKF produces smooth LPS but does not exhibit smooth colony phenotype or increased virulence. We also demonstrate that vaccination with strain RB51WboAKF results in enhanced protection against challenge with virulent *B. abortus* 2308 and *B. melitensis* 16M.

Materials and methods

Bacterial strains

B. abortus strains RB51, RB51WboA and 2308, and *B. melitensis* strain 16M were from our culture collection. The required plasmid constructs were prepared using *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA). Tryptic soy broth (TSB) and tryptic soy agar (TSA) were used as growth media for all bacterial strains. The growth medium was supplemented with chloramphenicol at 30 µg/ml for selection of bacteria harboring the plasmids. All protection experiments with virulent *Brucella* were performed in a BSL-3 facility approved for the select agents work.

Generation of recombinant strains RB51WbkF, RB51WboAKF and RB51WboAKD

Genes *wbkF* and *wbkD* were amplified using custom-designed primer-pairs by PCR. *B. abortus* 2308 genomic DNA was used as template. Forward primer (5'-TTTT*GGTACC*GGGCGTATGGTTGCGG) and reverse primer (5'-TTTT*CTCGAG*CGCTTCAGGAAGCTATGACC) containing *Kpn* I and *Xho* I restriction sites, respectively, were used to amplify *wbkD*. Similarly, forward primer (5'-TTTT*GGTACC*GAGCTTTGACATTATCCGTG) and reverse primer (5'-TTTT*CTCGAGG*TCATAGCTTCCTGAAG) containing *Kpn* I and *Xho* I restriction sites, respectively, were used to amplify *wbkF*. The amplified fragments were cloned in pGEM-T Easy plasmid (Promega, Madison, WI) and the integrity of the nucleotide sequences was confirmed by sequence analysis. The inserts were subsequently excised from the pGEM-T plasmids using the restriction enzymes specific to the respective restriction sites engineered into the primers. *wbkF* was subsequently cloned in the same sites of pBBR1Trc and pBBR/wboA [20,21]. wbkD was cloned in the same sites of pBBR1Trc [20,21]. The resulting plasmids, pBB1WbkF (*wbkF*), pBB1/wboAKF (*wboA* and *wbkF*) and pBB1/wboAKD (*wboA* and *wbkD*) were electroporated into strain RB51 as per previously

described procedure [22] to generate strains RB51WbKF, RB51WboAKF and RB51WboAKD, respectively.

SDS-PAGE and Western blotting

SDS-PAGE and Western blot analyses were carried out as previous described [2]. Briefly, antigen extracts of RB51, RB51WboA, RB51WboAKF, RB51WboAKD and *B. neotomae* were separated electrophoretically on a 12.5% denaturing polyacrylamide gel. The gel was subsequently stained with Coomassie Brilliant Blue. For Western blotting, the separated antigens were transferred onto a nitrocellulose membrane. Following membrane blocking in 5% skim milk for 3 hrs, the membrane was reacted with appropriately diluted *Brucella* O-PS specific rat monoclonal antibody, Bru-38 [4]. Horseradish peroxidase labelled-secondary antibody (KPL, Gaithersburg, MD) followed by the development of the enzymatic reaction by a colorimetric substrate (TMB substrate, KPL, Gaithersburg, MD) were used to detect the binding of the primary antibody.

Immuno-electron microscopy

Freshly grown cultures of *B. neotomae* and strains RB51 and RB51WboAKF were used. The bacterial cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer. They were then pelleted in 2% agarose (Sigma-Aldrich), dehydrated with a graded ethanol series to 85% ethanol and infiltrated with LR White resin. Polymerization was done overnight at 53°C in flat embedding molds (Ted Pella, Inc.). Sections were cut on a Reichert-Jung Ultracut E ultramicrotome and collected on 100 mesh formvar-coated nickel grids. Grids were incubated on 50 mM glycine for 30 mins and then on blocking solution (0.1 M sodium phosphate buffer containing 0.5% Aurion BSA-c™, 1% normal goat serum, and 0.2% Tween 20) for 1 hr. Primary antibody incubation was done overnight at 4°C with a 1:100 dilution of the rabbit anti-*B. neotomae* LPS antibody in blocking solution. Grids were rinsed seven times and incubated on goat anti-rabbit secondary antibody conjugated with 10 nm gold in blocking solution for 90 mins. Grids

were washed six times and stained with 2% uranyl acetate. Images were acquired on a FEI Tecnai G^2 20 electron microscope equipped with a LaB_6 source and operating at 100 kV.

Extraction of LPS

Total LPS was extracted from live *B. neotomae*, RB51, RB51WboA and RB51WboAKF by butanol-water method as previously described [23,24]. Briefly, freshly grown bacterial cultures were pelleted by centrifugation and suspended in 0.85% NaCl at a concentration of 0.25 g wet weight/ml. An equal volume of water saturated butanol was added with constant mixing for 15 mins at 4ºC. The aqueous phase was collected by centrifugation at 35,000×g for 20 mins. The LPS was precipitated using 4 volumes of cold methanol. The precipitate was dissolved in 0.1 M Tris buffer (pH 8) containing 2% SDS and 2% beta-mercaptoethanol and heated for 5 mins at 100ºC followed by incubation with proteinase K at 56ºC. LPS was finally precipitated by cold methanol and dissolved in water. Purity of the extracted LPS was confirmed by SDS-PAGE followed by silver staining of the gel. The extracted *B. neotomae* smooth LPS was used as antigen in indirect ELISA.

LPS analysis

A 12.5% and a 15% denaturing polyacrylamide gel was used for Western blot analysis and silver staining, respectively. The extracted LPSs were separated electrophoretically in sodium dodecyl sulfate-polyacrylamide gel. For Western blotting, the separated LPSs were transferred onto a nitrocellulose membrane. Following membrane blocking in 5% skim milk for 3 hrs, the membrane was reacted with appropriately diluted *B. neotomae* LPS-specific antibody. Horseradish peroxidase labelled-secondary antibody (KPL, Gaithersburg, MD) followed by the development of the enzymatic reaction by a colorimetric substrate (TMB substrate, KPL, Gaithersburg, MD) was used to detect the binding of the primary antibody. Silver staining of the polyacrylamide gel was performed using the Bio-RAD Silver Stain Plus kit according to the manufacturer's directions.

Polymyxin B sensitivity assay

Polymyxin B (PmB) sensitivity assay was performed as described previously [1,25]. Briefly, RB51, RB51WboAKF and *B. neotomae* were grown to log phase, in TSB for RB51 and *B. neotomae* and in TSB supplemented with 30 µg/ml chloramphenicol for RB51WboAKF. The bacterial cultures were pelleted by centrifugation at 4,200×g, resuspended in 10 mM phosphate buffer (pH 7.2) at approximately 1×10^4 CFU/ml, and incubated for 1 hr with different concentrations of polymyxin B. Following incubation, the cell suspensions were diluted 1:10 and 1:100 in 10 mM phosphate buffer and 50 µl was plated on TSA plates. The plates were incubated at 37°C for 5 days and the bacterial colonies on each plate were counted. Average results of three assays were expressed as percentages of the *Brucella* surviving in the absence of polymyxin incubation.

Vaccine preparation

B. abortus RB51, RB51WboA and RB51WboAKF were grown in liquid media to mid log phase, and aliquots of 1 ml were prepared and stored at -80ºC until further use. The number of bacteria in the aliquots was enumerated by performing ten-fold serial dilutions and plating them on TSA plates for RB51 and TSA plates supplemented with 30 µg/ml chloramphenicol for RB51WboA and RB51WboAKF. The plates were incubated at 37°C for 3-4 days and the bacterial CFUs were counted.

Persistence in mouse organs

Female BALB/c mice of 4 to 6 weeks of age were used for the studies. Mice were purchased from a commercial source (Harlan Laboratories, USA), and housed in cages with microisolator tops at 4 mice per cage. Feed and water were provided ad libitum. Housing conditions included standard 12 hrs light/dark cycle, controlled humidity (55%) and room temperature (22ºC). Mice were allowed to acclimatize to the new environment for 1 week before starting the experiments.

Groups of twelve mice each were inoculated by intra-peritoneal (i.p.) route with 2×10^8 CFU of live RB51 or RB51WboAKF. Three mice from each group were euthanized by CO_2 asphyxiation followed by cervical dislocation on days 1, 7, 21 and 28 post-immunization (p.i.) and their spleens, livers, and lungs were aseptically collected. The *Brucella* CFUs in the organs were enumerated as previously described [4]. Briefly, the tissues were homogenized with a tissue micer and were serially diluted 10-fold in TSB. Fifty µl from each serial dilution was plated on TSA plates for RB51 and on TSA plates containing 30 µg/ml chloramphenicol for RB51WboAKF. The plates were incubated for up to 5 days at 37ºC. The bacterial CFUs were enumerated.

Mice immunizations

Groups of 4 mice each were immunized by i.p. inoculation with 2×10^8 CFU of live RB51, RB51WboA or RB51WboAKF. A group was inoculated with saline only as a control. At 3 and 6 weeks p.i., blood from anesthetized mice was collected by puncture of retro-orbital plexus. The sera was separated from the clotted blood and was stored at -20°C until further use for antibody-mediated immune response analysis by indirect ELISA. Also, at 6 weeks p.i., the mice were anesthetized by CO_2 asphyxiation followed by cervical dislocation and their spleens were aseptically collected. The splenocytes were harvested from the spleens and used for the analysis of cell-mediated immune responses.

Indirect ELISA

Levels of RB51-specific and *B. neotomae* LPS-specific serum immunoglobulin M (IgM), IgG, as well as IgG1, IgG2a, IgG2b and IgG3 isotypes were determined using indirect ELISA [17,24,26]. RB51 was heat-killed by incubation at 65°C for 1 hr. The antigens were diluted in carbonate buffer (pH 9.6), to a final concentration of 1×10^9 CFU-equivalent of killed RB51/ml and 1 in 10 for *B. neotomae* LPS. These diluted antigens were plated in the wells (100 µl/well) of polystyrene plates (Nunc-Immunoplate with maxisorp surface). The plates were incubated overnight in 4°C. The plates were washed four times with wash

buffer (TBS at pH 7.4, 0.05% Tween 20) and incubated for 1 hr at 37°C with blocking buffer (5% skim milk in TBS). Diluted mouse sera (1:200 in blocking buffer) were added in duplicates to the wells (50 µl/well). After 4 hrs, the plates were again washed with wash buffer. Appropriately diluted anti-mouse horseradish peroxidase labelled isotype specific conjugates (Southern Biotechnology Associates Inc, Birmingham, Alabama) were added to the wells (50 µl/well). Following 1 hr of incubation at room temperature, the plates were washed with wash buffer to remove any unbound antibody. Substrate solution (TMB Microwell peroxidase substrate; KPL, Gaithersburg, MD) was added to each well (100 µl/well). The plates were incubated for 20 mins in the dark and the enzyme reaction was stopped by 100 µl of stop solution (0.185 M sulfuric acid). Finally, a microplate reader (Molecular devices, Sunnyvale, CA) was used to record the absorbance at 450 nm.

Culture of splenocytes

Single cell suspension of the splenocytes was prepared as per previously described procedure [24]. The erythrocytes were lysed using ACK lysis buffer and the splenocytes were cultured in triplicates in 96-well flat-bottomed culture plates, 5×10^5 cells/well for the quantification of cytokines and 10^6 cells/well for the analysis of antigen-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cells. The splenocytes were cultured in the presence of different stimulants: 10^7 CFU-equivalents of RB51 and RB51WboAKF. Cells stimulated with 2.5 µg/ml of concanavalin A (ConA) and plain media were used as controls.

Quantification of cytokines

The splenocytes were cultured with different stimulants and controls (as described above in culture of splenocytes) for 5 days in a humidified incubator with 5% CO_2 at 37°C and their supernatants were collected. The concentrations of the cytokines IL-2, GM-CSF, IFN-γ, TNF-α, IL-4, IL-5, IL-10 and IL-12p70 were determined using Bio-RAD Bio-Plex ProTM Mouse Cytokine Th1/Th2 Assay according to the manufacturer's instructions.

Flow cytometric analysis of antigen-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cells

A previously described procedure was followed for intracellular IFN-γ staining [24,27]. The splenocytes were cultured with different stimulants and controls (as described above in culture of splenocytes) for 8 hrs in a humidified incubator with 5% CO_2 at 37°C. Brefelin A (Golgistop; Pharmingen) was added to the wells and the plates were incubated for another 8 hrs. Cells from each treatment were suspended in FACS buffer (PBS containing 1% BSA and 0.2% sodium azide). They were incubated for 30 mins in ice with appropriately diluted FITC-conjugated anti-mouse CD8 antibody (BD Pharmigen, clone53-6.7) and APC-conjugated anti-mouse CD4 antibody (BD Pharmigen, clone L3T4-RM 4.5). Following three washes with FACS buffer, the cells were stained for intracellular IFN-γ with PE-conjugated rat anti-mouse IFN-γ antibody using the cytofix/cytoperm (Pharmingen) using manufacturer's instructions. As an isotype control, cells stained with PE-conjugated rat IgG1 antibody were used. The cells were acquired on BD FACS Canto II™ Flow cytometer (BD Biosciences, CA, USA). The data were analyzed using BD FACSDIVA version 6 software (BD Biosciences, CA, USA) and the proportion of $CD4^+$ and $CD8^+$ T cells that secreted IFN-γ was determined.

Quantification of serum cytokines

Groups of three mice each were infected with 2×10^8 CFU of live RB51 or RB51WboAKF. A group of mice inoculated with saline alone served as the negative control. Mice from all the groups were bled by retro-orbital puncture at days 1, 7 and 28 p.i. The serum was separated from the clotted blood and concentrations of the cytokines IL-2, GM-CSF, IFN-γ, TNF-α, IL-4, IL-5, IL-10 and IL-12p70 were determined using Bio-RAD Bio-Plex ProTM Mouse Cytokine Th1/Th2 Assay according to the manufacturer's instructions.

Spleen histology

Groups of six mice each were infected with 2×10^8 CFU of live RB51 or RB51WboAKF. A group of mice inoculated with saline was used as control. Two mice from each group were euthanized at days 1, 7 and 28 p.i. and their spleens were aseptically collected. The spleens were then weighed, and immersed in 10% neutral-buffered formalin, embedded, sectioned, mounted on microscopic slides, and stained with hematoxylin and eosin. The stained slides were digitized using an Aperio Digital Slide Scanner (Leica) and slides were analyzed by a board-certified pathologist (Grant N. Burcham). They were annotated using the ScanScope software (Leica).

Protection experiments

Protection experiments were performed at Virginia Tech in an ABSL-3 facility that was approved for work with select agents. Female BALB/c mice of 4-6 weeks of age (Harlan Laboratories, USA) were used for the studies. Mice were housed in individually ventilated cages with high-efficiency particulate arresting-filtered air. Feed and water were provided ad libitum. Housing conditions included standard 12 hrs light/dark cycle, controlled humidity (55%) and room temperature (22^oC). After 1 week of acclimatization, mice were administered with vaccine or control formulation.

Groups of ten mice each were vaccinated by i.p. inoculation with 2×10^8 CFU of live RB51, RB51WboA or RB51WboAKF. Ten mice inoculated with saline alone served as a control. Six weeks p.i, five mice in each group were challenged by i.p. inoculation with 5×10^4 CFU of *B. abortus* 2308 and the remaining five mice in each group were challenged with 5×10^4 CFU of *B. melitensis* 16M. Two weeks post-challenge, the mice were euthanized and the bacterial burden in their spleens was enumerated as previously described [4].

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. ELISA absorbance values, cytokine concentrations and flow cytometry data were analyzed for differences among groups by performing one-way analysis of variance (ANOVA) followed by Bonferroni test. The protection data was analyzed using student *t*-test modified for unequal variances between groups.

Ethics statement

The protocols of the mice experiments performed in this study were approved by the Institutional Animal Care and Use Committees at Purdue University (Approval # 1112000488) and Virginia Tech (Approval # CVM-10-048). The animal studies were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Blood was collected from the retro-orbital plexus from mice under anesthesia. For anesthetizing mice, regulated concentration of anesthetic mixture (oxygen and isoflurane) was administered via a commercially available rodent anesthesia machine (Vetamac, Inc., Rossville, Indiana). Following blood collection, a drop of proparacaine hydrochloride ophthalmic solution (Bausch & Lomb, Tampa, Florida) was placed on the eye to reduce pain. Mice infected with virulent *B. abortus* 2308 do not develop clinical disease or exhibit any signs of suffering for the duration of the experiments conducted in this study. Therefore, no humane endpoints were utilized for the mice in this study.

Results

O-Polysaccharide expression by strain RB51WboA overexpressing the wbkF gene

Strain RB51 was electroporated with plasmid pBB1/wboAKF, containing the functional *wboA* and *wbkF* genes from strain 2308, to generate strain RB51WboAKF. Five of the

plasmid-harboring recombinant colonies were selected for determination of O-PS expression. Western blot analysis with Bru-38 showed that all five RB51WboAKF colonies contained smooth LPS (Fig. 5.1A). The reactivity profile of the RB51WboAKF recombinants was similar to that of *B. neotomae*. In contrast, strain RB51WboAKD had a similar reactivity pattern as that of strain RB51WboA (data not shown). In strain RB51WboA, Bru-38 reacted with a few bands in the 45 kDa range, which was similar to previous descriptions [17] (Fig. 5.1A). As expected, RB51 did not show any reactivity with Bru-38 [4] (Fig. 5.1A). Strain RB51WbkF also did not react with Bru-38 (data not shown). One of the RB51WboAKF recombinants was selected for all the further studies.

SDS-PAGE followed by Coomassie Brilliant Blue staining was used to visualize any differences in the protein profiles of the strains RB51 and RB51WboAKF. There was no apparent qualitative difference in the protein profiles of the two strains (Fig. 5.1B).

Immuno-electron microscopy was carried out to determine the location of the expressed O-PS in bacterial cells. Immuno-electron microscopy of thin sections of RB51WboAKF revealed the presence of O-PS mostly on the surface of bacterial cells (Fig. 5.2C). As expected, no O-PS was detected in strain RB51 (Fig. 5.2B) while the presence of O-PS in strain *B. neotomae* was predominantly detected on the cell surface (Fig. 5.2A).

Indirect ELISA with whole bacterial cells as antigen was performed to determine the level of O-PS expression on the surface of bacterial cells. As expected, RB51 and RB51WboA whole cells did not react with smooth LPS-specific antibody (Fig. 5.3). In contrast, both strain RB51WboAKF and *B. neotomae* exhibited a higher level of reactivity with anti-smooth LPS antibody when compared with RB51 and RB51WboA strains; however, *B. neotomae* reacted with all tested dilutions of the smooth LPS-specific antibody at significantly higher levels when compared with strain RB51WboAKF (Fig. 5.3).

LPS analysis

In order to assess the degree of roughness of strain RB51WboAKF, the electrophoretic profile of the LPSs were examined by Western blotting and silver staining (Fig. 5.4A and B). Western blot analysis of LPS from RB51WboAKF revealed a reactive smear that

extended from 75kDa to 25kDa (Fig. 5.4A). In contrast, LPS from RB51 and RB51WboA did not show any reactivity with smooth LPS-specific antibody (Fig. 5.4A). LPS from *B. neotomae* exhibited positive reactivity with anti-smooth LPS antibody and a smear that extended from >100kDa to <20kDa was visible on Western blotting (Fig. 5.4A).

On silver staining of the polyacrylamide gel, a major low-molecular weight band of the same size (<10kDA) was visible in the LPSs of all the four strains indicating that all the strains exhibit a complete core (Fig. 5.4B). Additionally, a high-molecular weight band (≈23kDA) and a low-molecular weight band (≈11kDA) were present in the LPS profiles of *B. neotomae* and RB51WboAKF but absent in RB51 and RB51WboA (Fig. 5.4B). A band of intermediate size (≈18kDA) was present in the LPSs of strains RB51, RB51WboAKF and RB51WboA but absent in LPS of *B. neotomae* (Fig. 5.4B).

Sensitivity to the bactericidal effect of polymyxin B

PmB at concentrations ranging from 2 µg/ml to 50 µg/ml was used to test the ability of strain RB51WboAKF to resist bactericidal killing. Strain RB51WboAKF was less sensitive to the bactericidal effect of PmB when compared to strain RB51 at concentrations ≥ 10 µg/ml (Fig. 5.5). The difference was more apparent at higher concentrations (20-50 µg/ml) of PmB (Fig. 5.5). As expected, tested concentrations of PmB had the least effect on *B. neotomae* which exhibited enhanced survival percentage when compared to the strains RB51 and RB51WboAKF (Fig. 5.5).

Persistence of strain RB51 and RB51WboAKF in mice.

Persistence of strains RB51 and RB51WboAKF in mice organs was compared in order to assess the attenuation characteristic of strain RB51WboAKF. There was no difference in the persistence pattern of RB51 and RB51WboAKF in spleens, livers and lungs of the vaccinated mice at days 1, 7, 21 and 28 p.i. (Fig. 5.6). Both the strains were cleared from the mice livers and lungs and persisted at very low levels in spleens of mice at day 28 p.i. (Fig. 5.6).

Induction of specific antigen-antibody responses in mice

Specific antigen-antibody responses induced in vaccinated mice were determined by indirect ELISA. Higher levels of LPS-specific total IgG antibodies were detected in the serum of mice vaccinated with RB51WboA and RB51WboAKF when compared with RB51 vaccinated and saline-inoculated mice (Fig. 5.7). LPS-specific IgG levels were significantly higher in RB51WboAKF vaccinated mice when compared with those vaccinated with RB51WboA (Fig. 5.7). Mice vaccinated with RB51WboAKF also had significantly higher levels of LPS-specific IgM, IgG2a, IgG2b and IgG3 at 3 and 6 weeks p.i. and LPS-specific IgG1 at 3 weeks p.i. when compared with RB51 vaccinated and saline-inoculated mice group (Fig. 5.7). In contrast, significantly higher levels of LPS-specific IgG2a and IgG3 antibodies were detected in mice vaccinated with RB51WboA only at 6 weeks p.i. when compared with the RB51 vaccinated and unvaccinated groups of mice (Fig. 5.7). The levels of LPS-specific IgG2a and IgG3 were significantly higher in the serum of RB51WboAKF vaccinated mice than those vaccinated with RB51WboA (Fig. 5.7).

All the vaccinated groups of mice (RB51, RB51WboA and RB51WboAKF) had significantly higher levels of RB51-specific IgG, IgM, IgG2a, IgG2b and IgG3 when compared with the saline inoculated controls (Fig. 5.8). Only the levels of RB51-specific IgG2a were significantly higher in the serum of RB51WboAKF vaccinated mice when compared with RB51WboA vaccinated group of mice (Fig. 5.8).

Histology of spleens

RB51WboAKF vaccinated mice had higher spleen weights (453.33 ± 20.54 mg) at day 7 p.i. when compared to RB51 vaccinated mice (403.33 ± 26.25 mg) ($P < 0.05$). Splenic histology in saline-inoculated and RB51 vaccinated mice was considered normal at day 7 p.i. (Fig. 5.9A and B). In contrast, markedly increased extramedullary hematopoiesis (EH) was observed in RB51WboAKF vaccinated mice at day 7 p.i. (Fig. 5.9C). At day 28, a trend towards increase in the number of germinal centers per spleen was observed in

RB51WboAKF vaccinated mice spleens when compared with the spleens of RB51 vaccinated mice.

Analysis of serum cytokines

Vaccination of mice with RB51 and RB51WboAKF resulted in significantly higher levels of IL-12 and IFN-γ at day 1 and TNF-α at day 7 p.i. when compared with the saline-inoculated mice (Fig. 5.10). The level of IL-12 and IFN-γ was significantly higher at day 1 p.i. in the serum of WboAKF immunized mice than those immunized with RB51 (Fig. 5.10). Moreover, significantly increased levels of GM-CSF at day 27 p.i. and IL-10 at days 1 and 7 p.i. were detected in the serum of mice vaccinated with RB51WboAKF when compared with RB51 vaccinated and saline inoculated mice groups (Fig. 5.10). The concentrations of IL-4 and IL-5 were below the limit of detection in the vaccinated groups of mice as well as the saline-inoculated group (data not shown).

Induction of specific cell-mediated responses in mice

Specific cell-mediated responses were analyzed at 6 weeks p.i. in the vaccinated mice by determining the number of IFN-γ secreting $CD4^+$ and $CD8^+$ T cells as well as by quantifying cytokines secreted by splenocytes upon in vitro stimulation with specific antigens.

Upon in vitro stimulation with RB51 and RB51WboAKF, higher proportions of IFN-γ secreting $CD4^+$ and $CD8^+$ T cells were detected in all the vaccinated groups of mice but not the saline-inoculated group when compared with the corresponding unstimulated controls (Fig. 5.11A and B). When stimulated with RB51WboAKF, significantly higher proportions of IFN- γ secreting $CD4^+$ and $CD8^+$ T cells were detected in RB51WboAKF vaccinated mice when compared to the other vaccinated groups of mice (Fig. 5.11).

Upon in vitro stimulation with RB51 and RB51WboAKF, splenocytes of all groups of vaccinated mice, but not the saline-inoculated group, secreted significantly increased levels of IFN-γ, GM-CSF and IL-10 when compared to the corresponding unstimulated controls

(Fig. 5.12). In contrast, concentrations of IL-4 and IL-5 in the culture supernatants of all groups of mice were below the detection limit and not different from the corresponding unstimulated controls (data not shown). Stimulation with RB51WboAKF resulted in the secretion of significantly higher levels of IFN-γ and IL-10 by the splenocytes of RB51WboAKF vaccinated group of mice than the other vaccinated mice groups (Fig. 5.12). Also, upon in vitro stimulation with RB51 and RB51WboAKF, significantly higher levels of TNF-α were detected in the culture supernatants of mice vaccinated with RB51WboAKF and RB51WboA, but not the saline-inoculated group, when compared with their unstimulated controls; the level of TNF-α was significantly higher in the RB51WboAKF vaccinated mice group when compared with other vaccinated groups of mice (Fig. 5.12). In contrast, only stimulation with RB51 resulted in the secretion of higher levels of TNF-α by splenocytes of RB51 vaccinated mice when compared to the corresponding unstimulated control (Fig. 5.12). In vitro stimulation with RB51 resulted in the secretion of higher levels of IL-2 by splenocytes of RB51 and RB51WboAKF vaccinated groups of mice, but not the saline-inoculated group, when compared with the corresponding unstimulated controls (Fig. 5.12); however, higher levels of IL-2 were only detected in the culture supernatants of mice vaccinated with RB51WboAKF, after in vitro stimulation with RB51WboAKF, when compared to the corresponding unstimulated control (Fig. 5.12).

Enhanced protection against virulent challenge infection

To assess the protection afforded by different vaccine strains in a mouse model of brucellosis, bacterial CFUs were calculated in the vaccinated groups of mice two weeks post-challenge with virulent *Brucella* species. Vaccination with RB51, RB51WboA and RB51WboAKF resulted in significant reduction of the splenic bacterial load following challenge with virulent *B. abortus* 2308 and *B. melitensis* 16M when compared with the saline inoculated group of mice (Fig. 5.13A and B). Following challenge with virulent *B. abortus* 2308 and *B. melitensis* 16M, RB51WboAKF immunized group of mice showed

the highest reduction in the splenic bacterial load when compared with RB51 and RB51WboA vaccinated groups (Fig. 5.13).

Discussion

The studies in this paper demonstrate that overexpression of *wbkF* in strain RB51WboA leads to the expression of O-PS similar to the smooth *Brucella* strains and enhanced protection against *B. abortus* 2308 and *B. melitensis* 16 M. Our immunoelectron study as well as indirect ELISA using whole live bacterial cells as antigens suggests that the synthesized O-PS by the strain RB51WboAKF is present mostly on the bacterial cell surface (Fig. 5.2 and 5.3). However, strain RB51WboAKF still exhibited rough strain characteristics as evidenced by agglutination with acriflavin. Also, silver stained profile of the extracted LPS from strain RB51WboAKF was partly similar to that of both rough and smooth strains (Fig. 5.4B). A previous study has described a strain of *B. melitensis* (strain EP) which simultaneously exhibits smooth and rough characteristics [28]. This type of 'smooth-rough' phenotype of strain EP was attributed to the presence of fewer O-PS chains and higher amounts of rough-LPS when compared to the strain 16M [28]. Similarly, reduced level of reaction of RB51WboAKF bacterial cells with smooth LPS-specific antibody when compared with *B. neotomae* indicates that fewer O-PS chains are expressed on RB51WboAKF cell surface. Fewer surface-expressed O-PS chains can also explain the enhanced sensitivity of strain RB51WboAKF to the bactericidal effect of polymyxin when compared to *B. neotomae* (Fig. 5.5). In case of RB51WboA, absence of positive reactivity with anti-smooth LPS antibody is expected as the O-PS is only expressed in the cytoplasm of the bacteria [17].

The complete biosynthetic pathway of *Brucealla* O-PS is yet to be determined. However, a pathway for it has been proposed based on the prediction of protein functions from comparisons with homologous proteins in other microorganisms and identification of rough *Brucella* phenotypes [2,15,29-32]. In the purported biosynthetic pathway of smooth LPS, WbkD and WbkF catalyzed steps lead to bactoprenol priming for N-formylperosamine polymerization by glycosyltransferases [29]. Consequently, *Brucella*

strains with mutations in *wbkF* or *wbkD* genes cannot produce O-PS [29]. *wbkD* encodes an epimerase/dehydratase that takes part in the synthesis of N-acetylquinovosamine [29,33] that has been found in the smooth LPS [3,7,34]. Previously, it has been proposed that N-acetylquinovosamine might act as a substrate for WbkF (undecaprenyl-glycosyltransferase) for the synthesis of bactoprenol primed N-acetylaminosugar [29]. In our study, overexpression of *wbkD* in strain RB51WboA did not change the level of O-PS expression when compared to that expressed by RB51WboA (data not shown). This finding suggests that a mere increase in the level of WbkD expression or quinivosamine does not affect O-PS expression. In contrast, increasing the level of WbkF in RB51WboA leads to enhanced O-PS expression (Fig. 5.1A) suggesting that overexpression of *wbkF* furnishes higher amounts of bactoprenol primed moieties for O-PS polymerization by *wboA* and other glycosyltransferases. As expected, overexpression of *wbkF* alone in RB51 did not lead to O-PS expression (data not shown) indicating that *wboA* encoded glycosyltransferase is required for O-PS polymerization.

The attenuation characteristic of strain RB51WboAKF did not differ from the parent strain RB51. Based on the increased resistance to polymyxin B treatment, we had expected that strain RB51WboAKF would persist longer in mice organs than strain RB51. The lack of any increase in the virulence characteristic of strain RB51WboAKF suggests that the attenuation of strain RB51 is because of mutations in multiple yet to be identified genes.

Vaccination of mice with strain RB51WboAKF resulted in the induction of increased IgG2a and IgG3 subisotype antibodies specific to the smooth LPS than other vaccinated and non-vaccinated groups (Fig 5.7). This indicates that a Th1 type of immune response was induced by RB51WboAKF vaccine. Previous studies have validated the contribution of Th1 immunity in protection against intracellular bacterial infections including brucellosis. Detection of significantly higher concentration of IFN-γ in the culture supernatants of antigen-specific splenocytes as well as significantly higher proportions of antigen-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cells in the RB51WboAKF vaccinated group of mice also indicates the induction of Th1 type of immunity. The presence of increased concentrations of TNF-α also suggests the development of Th1 effector cells.

Significantly enhanced TNF-α secretion might also play a role in the induction of splenomegaly and EH in RB51WboAKF vaccinated mice spleens. Pro-inflammatory cytokines such as TNF-α help in the recruitment of inflammatory cells leading to splenomegaly during *Brucella* infection [35]. Cytokines such as TNF-α and GM-CSF also increase myelopoiesis by supporting the growth and maturation of myeloid progenitors [36]. This enhanced production of mature myeloid cells helps in clearing pathogens from body [37].

RB51WboAKF vaccine conferred significantly higher protection against virulent *Brucella* species than strain RB51 and RB51WboA (Fig. 5.13). Both, T cell-mediated responses as well as O-PS specific antibodies, play a role in providing enhanced protection against brucellosis [9,13,29]. IFN-γ secreting T cells are implicated as the main effectors that provide acquired resistance against *Brucella* infection [9]. Previous studies show that antibodies to the O-PS including IgM, IgG2a, IgG2b and IgG3 can also mediate protection in mice [12,26,38,39]. The enhanced protection afforded by RB51WboAKF might be attributed to the anti-O-PS antibodies and/or to the enhanced cell-mediated immune responses. Further studies involving adoptive transfer experiments of O-PS-specific antibodies and/or immune T cells can characterize their specific contribution to the protective efficacy of RB51WboAKF.

In conclusion, our results suggest that RB51 carries mutations in several genes required for the synthesis of smooth LPS. Also, our study demonstrates that RB51WboAKF strain, while maintaining the attenuation characteristic of strain RB51, is more immunogenic and protective than strain RB51 and can be a more efficacious vaccine in natural hosts.

References

[1] Allen, C. A., Adams, L. G., Ficht, T. A., 1998. Transposon-derived *Brucella abortus* rough mutants are attenuated and exhibit reduced intracellular survival. Infect Immun 66, 1008-1016.

[2] Godfroid, F., Taminiau, B., Danese, I., Denoel, P., Tibor, A., Weynants, V., Cloeckaert, A., Godfroid, J., Letesson, J. J., 1998. Identification of perosamine synthetase gene of *Brucella melitensis* 16M and involvement of lipopolysaccharide O side chain in *Brucella* survival in mice and in macrophages. Infect Immun 66, 5485-5493.

[3] McQuiston, J. R., Vemulapalli, R., Inzana, T. J., Schurig, G. G., Sriranganathan, N., Fritzinger, D., Hadfield, T. L., Warren, R. A., Snellings, N., Hoover, D., Halling, S. M., Boyle, S. M., 1999. Genetic characterization of a Tn-5 disrupted glycosyltransferase gene homolog in *Brucella abortus* and its effect on lipopolysaccharide composition and virulence. Infect Immun 67, 3830-3835.

[4] Schurig, G. G., Roop, R. M. II., Bagchi, T., Boyle, S., Buhrman, D., Sriranganathan, N., 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 28, 171-188.

[5] Winter, A. J., Schurig, G. G., Boyle, S. M., Sriranganathan, N., Bevins, J. S., Enright, F. M., Elzer, P. H., Kopec, J. D., 1996. Protection of BALB/c mice against homologous and heterologous species of *Brucella* by rough strain vaccines derived from *Brucella melitensis* and *Brucella suis* biovar 4. Am J Vet Res 57, 677-683.

[6] Bundle, D. R., Cherwonogrodzky, J. W., Perry, M. B., 1987. The structure of the lipopolysaccharide O-chain (M antigen) and polysaccharide B produced by *Brucella melitensis* 16 M. FEBS Lett 216, 261-264.

[7] Caroff, M., Bundle, D. R., Perry, M. B., Cherwonogrodzky, J. W., Duncan, J. R., 1984. Antigenic S type lipopolysaccharide of *Brucella abortus* 1119-3. Infect Immun 46, 384-388.

[8] Eisenschenk, F. C., Houle, J. J., Hoffmann, E. M., 1995. Serum sensitivity of field isolates and laboratory strains of *Brucella abortus*. Am J Vet Res 56, 1592-1598.

[9] Yingst, S., Hoover, D. L., 2003. T cell immunity to brucellosis. Crit Rev Microbiol 29, 313-331.

[10] Araya, L. N., Winter, A. J., 1990. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infect Immun 58, 254-256.

[11] Ficht, T. A., Kahl-Mcdonagh, M. M., Arenas-Gamboa, A. M., Rice-Ficht, A. C., 2009. Brucellosis: the case for live, attenuated vaccines. Vaccine 27, D40-D3.

[12] Montaraz, J. A., Winter, A. J., Hunter, D. M., Sowa, B. A., Wu, A. M., Adams, L. G., 1986. Protection against *Brucella abortus* in mice with O-polysaccharide-specific monoclonal antibodies. Infect Immun 51, 961-963.

[13] Vitry, M. A., Mambres, D. H., Trez, C. D., Akira, S., Ryffel, B., Letesson, J. J., Muraille, E., 2014. Humoral immunity and $CD4^+$ Th1 cells are both necessary for a fully protective immune response upon secondary infection with *Brucella melitensis*. J Immunol 192, 3740-3752.

[14] Cloeckaert, A., Zygmunt, M. S., Guilloteau, L. A., 2002. *Brucella abortus* vaccine strain RB51 produces low levels of M-like O-antigen. Vaccine 20, 1820-1822.

[15] Zygmunt, M. S., Blasco, J. M., Letesson, J. J., Cloeckaert, A., Moriyon, I., 2009. DNA polymorphism analysis of *Brucella* lipopolysaccharide genes reveals marked differences in O-polysaccharide biosynthetic genes between smooth and rough *Brucella* species and novel species-specific markers. BMC Microbiol 9, 92-104.

[16] Vemulapalli, R., McQuiston, J. R., Schurig, G. G., Sriranganathan, N., Halling, S. M., Boyle, S. M., 1999. Identification of an *IS711* element interrupting the *wboA* gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. Clin Diag Lab Immunol 6, 760-764.

[17] Vemulapalli, R., He, Y., Buccolo, L. S., Boyle, S. M., Sriranganathan, N., Schurig, G. G., 2000. Complementation of *Brucella abortus* RB51 with a functional *wboA* gene results in O-antigen synthesis and enhanced vaccine efficacy but no change in rough phenotype and attenuation. Infect Immun 68, 3927-3932.

[18] Vemulapalli, R., Contreras, A., Sanakkayala, N., Sriranganathan, N., Boyle, S. M., Schurig, G. G., 2004. Enhanced efficacy of recombinant *Brucella abortus* RB51 vaccines against *B. melitensis* infection in mice. Vet Microbiol 102, 237-245.

[19] Adone, R., Muscillo, M., La Rosa, G., Francia, M., Tarantino, M., 2011. Antigen, immunologic and genetic characterization of rough strains *B. abortus* RB51, *B. melitensis* B115 and *B. melitensis* B18. PLOS ONE 6, e24073.

[20] Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., Peterson, K. M., 1994. pBBR1MCS: a broad-host-range cloning vector. Biotech 16, 800-801.

[21] Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M. 2nd., Peterson, K. M., 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166, 175-176.

[22] McQuiston, J. R., Schurig, G. G., Sriranganathan, N., Boyle, S. M., 1995. Transformation of *Brucella* species with suicide and broad host-range plasmids. Methods Mol Biol 47, 143-148.

[23] Phillips, M., Pugh, G. W., Deyoe, B. L., 1989. Chemical and protective properties of *Brucella* lipopolysaccharide obtained by butanol extraction. Am J Vet Res 50, 311-317.

[24] Moustafa, D., Garg, V. K., Jain, N., Sriranganathan, N., Vemulapalli, R., 2011. Immunization of mice with gamma-irradiated *Brucella neotomae* and its recombinant strains induces protection against virulent *B. abortus, B. melitensis* and *B. suis* challenge. Vaccine 29, 784-794.

[25] Martinez de Tejada, G., Pizzaro-Cerda, J., Moreno, E., Moriyon, I., 1995. The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. Infect Immun 63, 3054-3061.

[26] Elzer, P. H., Jacobson, R. H., Nielsen, K. H., Douglas, J. T., Winter, A. J., 1994. BALB/c mice infected with *Brucella abortus* express protracted polyclonal responses of both IgG2a and IgG3 isotypes. Immunol Lett 42, 145-150.

[27] Murali-Krishna, K., Altman, J. D., Suresh, M., Sourdive, D. J. D., Zajac, A. J., Miller, J., Ahmed, R., 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immun 8, 177-187.

[28] Bowden, R. A., Verger, J. M., Grayon, M., Limet, J. N., Dubray, G., 1993. Simultaneous expression of smooth and rough phase properties related to lipopolysaccharide in a strain of *Brucella melitensis*. J Med Microbiol 39, 363-370.

[29] Gonzales, D., Grillo, M. J., Miguel, M. J. D., Ali, T., Arce-Gorvel, V., Delrue, R. M., Conde-Alvarez, R., Munoz, P., Lopez-Goni, I., Iriarte, M., Marin, C. M., Weintraub, A., Widmalm, G., Zygmunt, M., Letesson, J. J., Gorvel, J. P., Blasco, J. M., Moriyon, I., 2008. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLOS ONE 3, e2760.

[30] Godfroid, F., Cloeckaert, A., Taminiau, B., Danese, I., Tibor, A., de Bolle, X., Mertens, P., Letesson, J. J., 2000. Genetic organization of the lipopolysaccharide O-antigen biosynthesis region of *Brucella melitensis* 16M (*wbk*). Res Microbiol 151, 655-668.

[31] Monreal, D., Grillo, M. J., Gonzalez, D., Marin, C. M., De Miguel, M. J., Lopen-Goni, I., Blasco, J. M., Cloeckaert, A., Moriyon, I., 2003. Characterization of *Brucella abortus* O-polysaccharide and core lipopolysaccharide mutants and demonstration that a complete core is required for rough vaccines to be efficient against *Brucella abortus* and *Brucella ovis* in the mouse model. Infect Immun 71, 3261-3271.

[32] Haag, A. F., Myka, K. K., Arnold, M. F. F., Caro-Hernandez, P., Ferguson, G. P., 2010. Importance of lipopolysaccharide and cyclic β-1,2-glucans in *Brucella*-mammalian infections. Int J Microbiol, doi: 10.1155/2010/124509.

[33] Kneidinger, B., Larocque, S., Brisson, J. R., Cadotte, N., Lam, J. S., 2003. Biosynthesis of 2-acetamido-2,6-dideoxy-L-hexoses in bacteria follows a pattern distinct from those of the pathways of 6-deoxy-L-hexoses. J Biochem 371, 989–995.

[34] Bowser, D. V., Wheat, R. W., Foster, J. W., Leong, D., 1974. Occurrence of quinovosamine in lipopolysaccharides of *Brucella* species. Infect Immun 9, 772–774.

[35] Roset, M. S., Ibanez, A. E., Filho, J. A. S., Spera, J. M., Minatel, L., Oliveira, S. C., Giambartolomei, G. H., Cassataro, J., Briones, G., 2014. *Brucella* cyclic β-1,2-glucan plays a central role in the induction of splenomegaly in mice. PLOS ONE 9, e101279.

[36] Cotterell, S. E., Engwerda, C. R., Kaye, P. M., 2000. *Leishmania donovani* infection of bone marroe stromal macrophages selectively enhances myelopoiesis by a mechanism involving GM-CSF and TNF-α. Blood 95, 1642-1651.

[37] Kim, C. H., 2010. Homeostatic and pathogenic extramedullary hematopoiesis. J Blood Med 1, 13-19.

[38] Elzer, P. H., Jacobson, R. H., Jones, S. M., Nielsen, K. H., Douglas, J. T., Winter, A. J., 1994. Antibody-mediated protection against *Brucella abortus* in BALB/c mice at successive periods after infection: variation between virulent strain 2308 and attenuated vaccine strain 19. Immunol 82, 651-658.

[39] Cloeckaert, A., Jacques, I., de Wergifosse, P., Dubray, G., Limet, J. N., 1992. Protection against *Brucella melitensis* or *Brucella abortus* in mice with immunoglobulin G (IgG), IgA, and IgM monoclonal antibodies specific for a common epitope shared by the *Brucella* A and M smooth lipopolysaccharides. Infect Immun 60, 312-315.

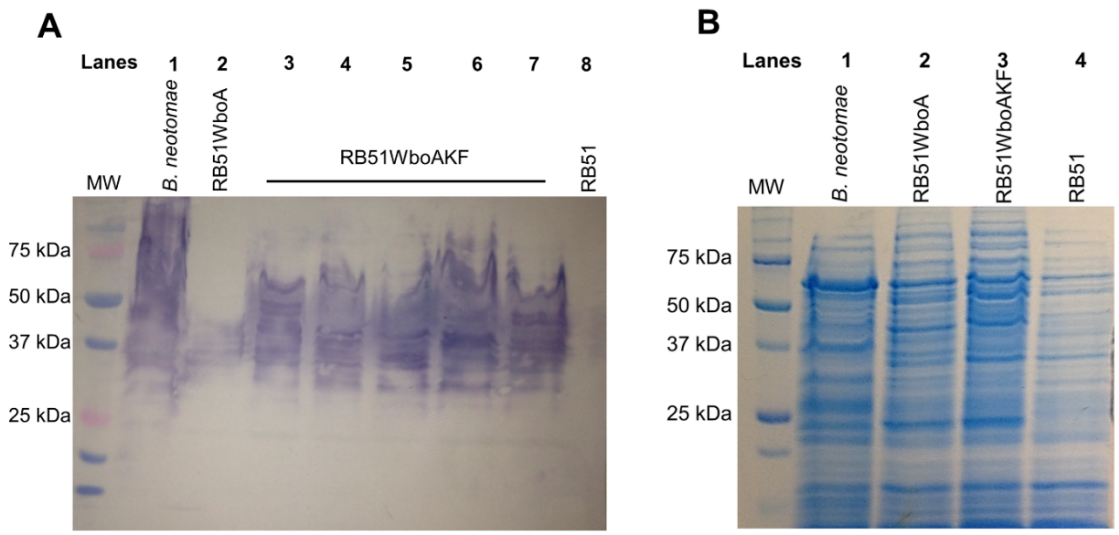

Figure 5.1. (A) Western blot analysis to detect O-polysaccharide expression by the strain RB51WboAKF and (B) Coomassie Brilliant Blue staining of the polyacrylamide gel to detect difference in the protein profiles of RB51, *B. neotomae*, RB51WboA and RB51WboAKF. Whole antigens of the strain RB51, recombinant strain RB51WboA, RB51WboAKF and *B. neotomae* were separated by 12.5% SDS-PAGE and analyzed by (A) Western blotting with O-polysaccharide specific monoclonal antibody, Bru-38. Lane 1: strain *B. neotomae*; lane 2: recombinant strain RB51WboA; lane 3-7: recombinant strain RB51WboAKF; lane 8: strain RB51. Whole antigens of the strain *B. neotomae*, RB51WboA, RB51WboAKF and RB51 were analyzed for any differences in the expressed protein profiles by (B) Coomassie Brilliant Blue stained polyacrylamide gel.

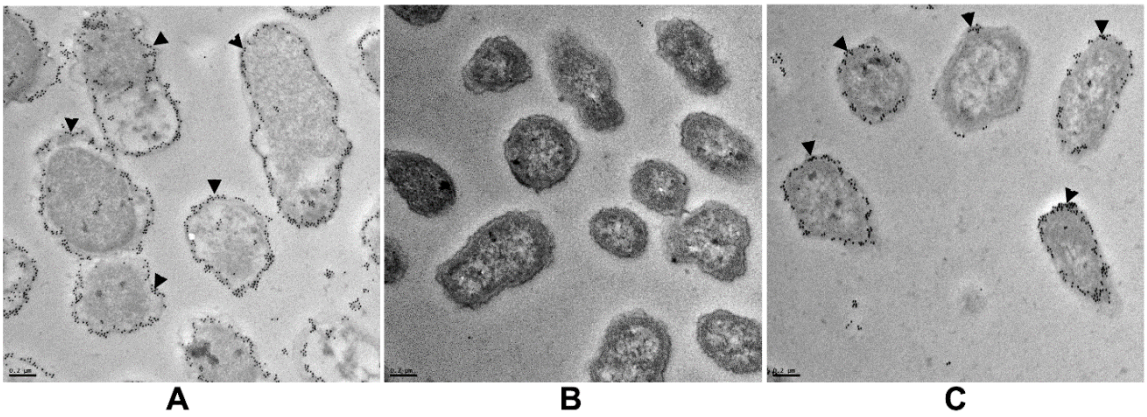

Figure 5.2. Detection of *Brucella* O-polysaccharide expression in strain RB51WboAKF by immunoelectron microscopy. Thin sections of (A) *B. neotomae*, (B) RB51 and (C) RB51WboAKF were reacted with anti-*B. neotomae* LPS followed by gold-labeled goat anti-rabbit IgG. The black arrows in panel A and C indicate the gold-labeled particles. Bars = 0.2 µm.

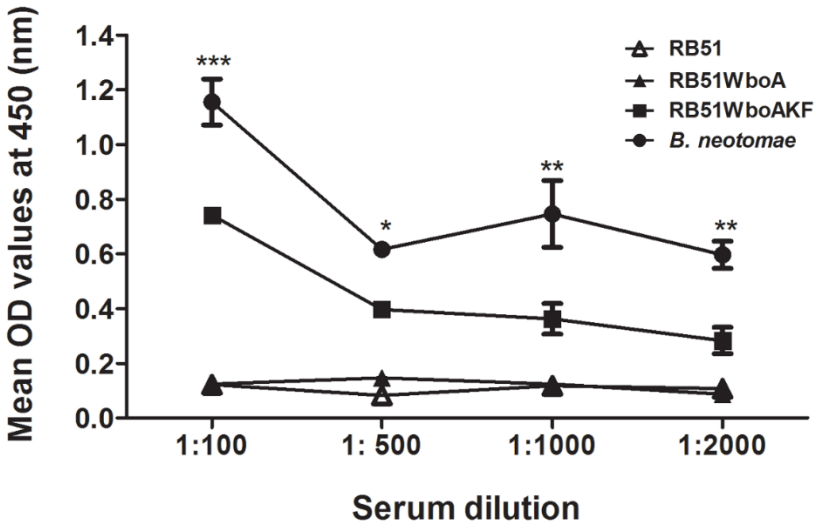

Figure 5.3. ELISA detection of O-polysaccharide expression on the cell surface of strain RB51WboAKF. RB51, RB51WboA, RB51WboAKF and *B. neotomae* whole cells (10^8 CFU/well) were reacted with specific dilutions of anti-*B. neotomae* LPS antibody. The results are shown as mean ± standard deviation of the optical density (OD) at 450 nm of the color developed. Asterisks indicate statistically significant differences from strain RB51WboAKF. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. OD, optical density.

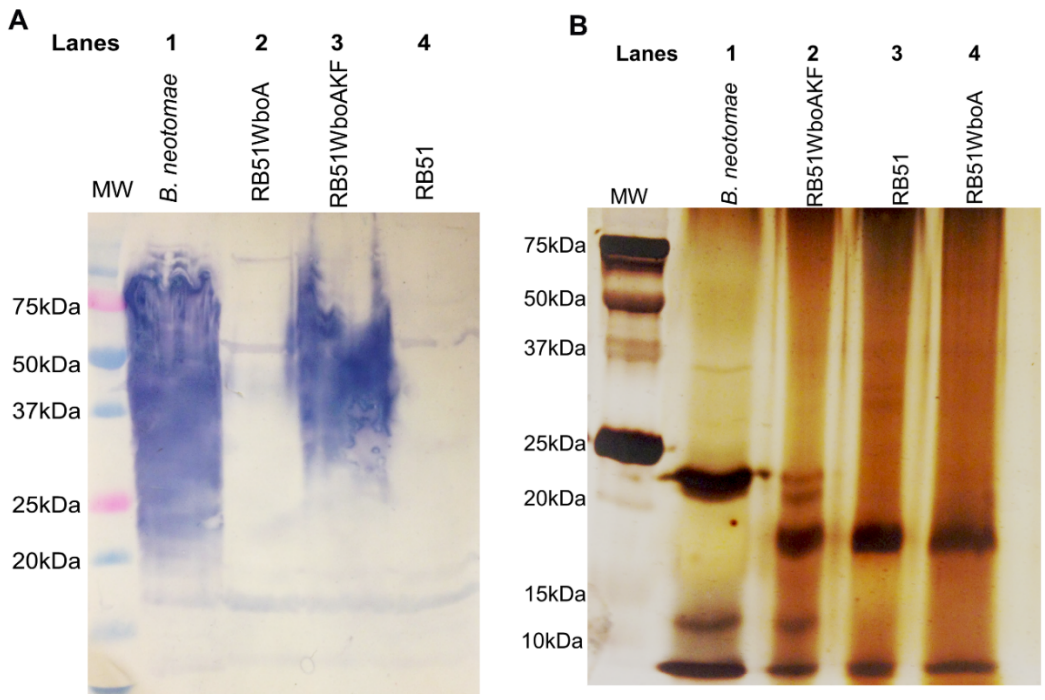

Figure 5.4. (A) Western blot analysis and (B) silver staining of the polyacrylamide gel to detect the differences in the LPS profiles of strains RB51, *B. neotomae*, RB51WboA and RB51WbkA. LPSs extracted from strain RB51, RB51WboA, RB51WboAKF and *B. neotomae* were separated by SDS-PAGE and analyzed by (A) Western blotting; Lane 1: strain *B. neotomae*; lane 2: recombinant strain RB51WboA; lane 3: recombinant strain RB51WboAKF; lane 4: strain RB51, and (B) silver staining; Lane 1: strain *B. neotomae*; lane 2: recombinant strain RB51WboAKF; lane 3: strain RB51; lane 4: recombinant strain RB51WboA.

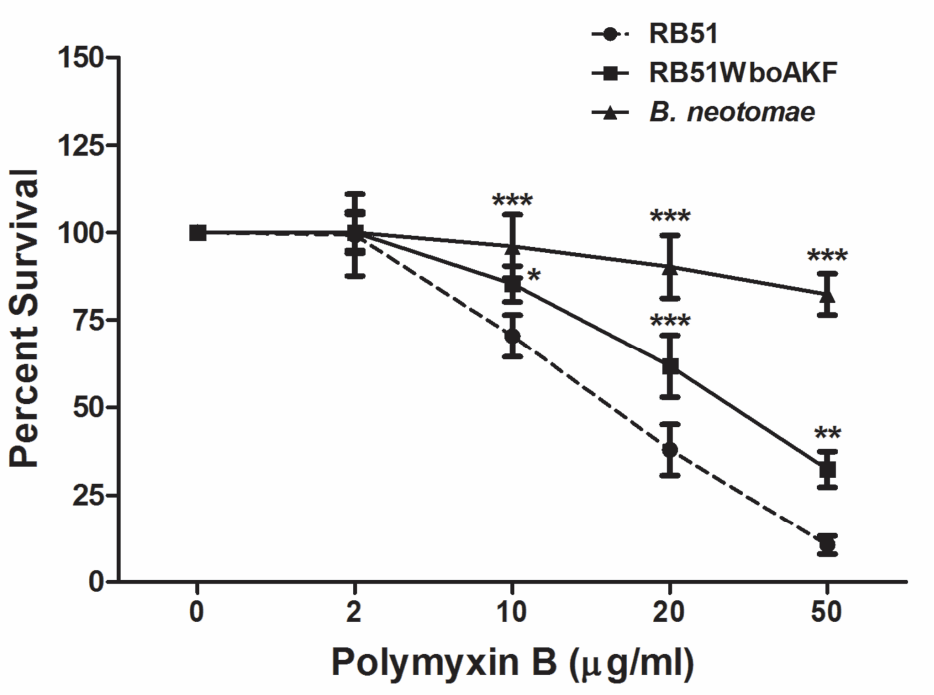

Figure 5.5. Determination of the bactericidal effect of polymyxin on strains RB51, RB51WboAKF and *B. neotomae*. Polymyxin-sensitivity assay was performed as described in Materials and methods. Average results from three assays are expressed as percentage of the bacteria surviving in the absence of polymyxin. Asterisks indicate statistically significant differences from the RB51 strain. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

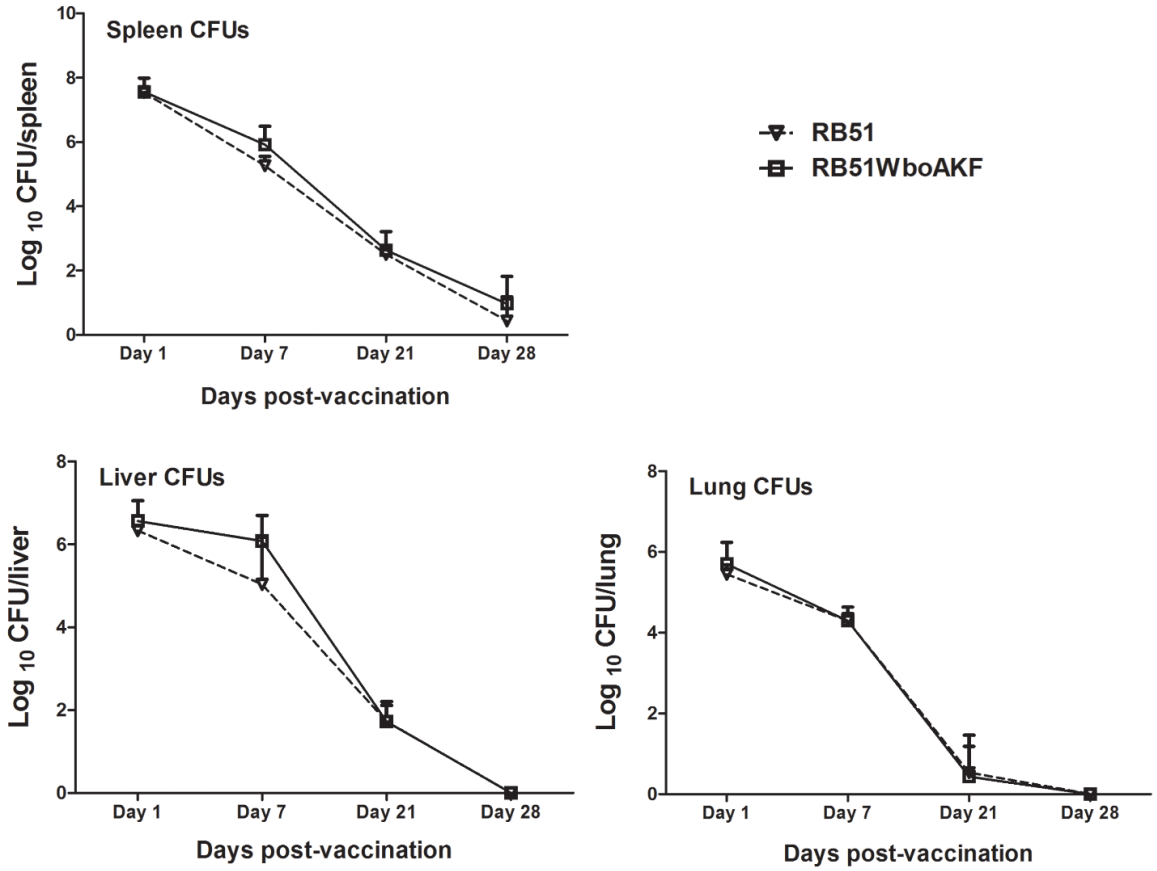

Figure 5.6. Determination of bacterial persistence of strain RB51WboAKF in mice. Mice spleens, livers and lungs were collected at days 1, 7, 21 and 28 after vaccination with live RB51 and live RB51WboAKF. The *Brucella* CFUs in these organs were determined as described in Materials and methods. Results are shown as mean ± standard deviation (n=3) of the log CFU of *Brucella* recovered from each organ.

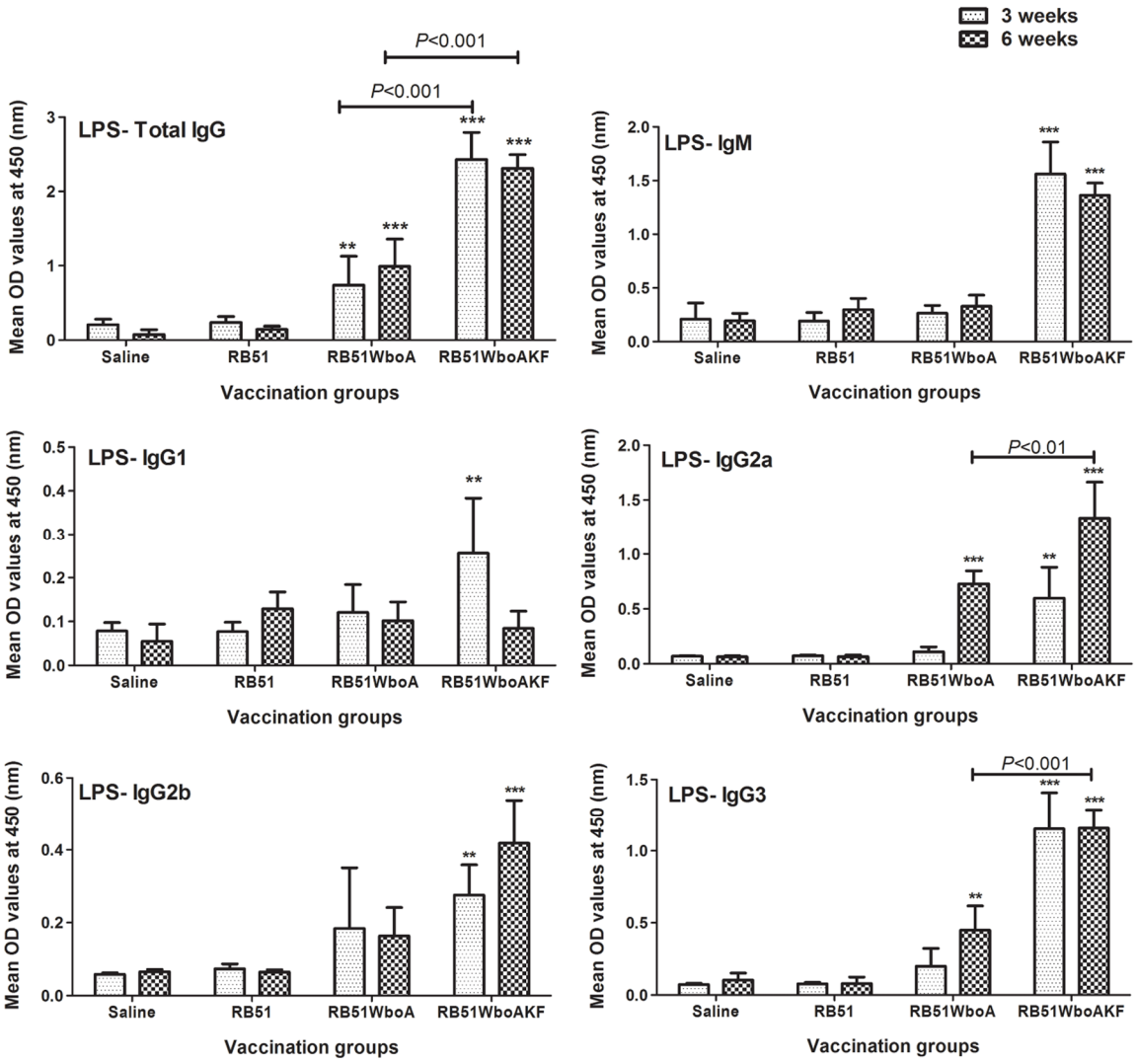

Figure 5.7. Detection of *B. neotomae* LPS-specific antibodies in serum of mice vaccinated with live recombinant RB51WboAKF. Mice were vaccinated with live RB51, RB51WboA, RB51WboAKF, or inoculated with saline. Serum samples were collected at 3 and 6 weeks after vaccination, were diluted 1 in 200 and assayed for the presence of *B. neotomae* LPS-specific IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies by indirect ELISA. Results are shown as mean ± standard deviation (n=4) of absorbance of the color developed. Asterisks indicate statistically significant differences from the corresponding saline group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. OD, optical density.

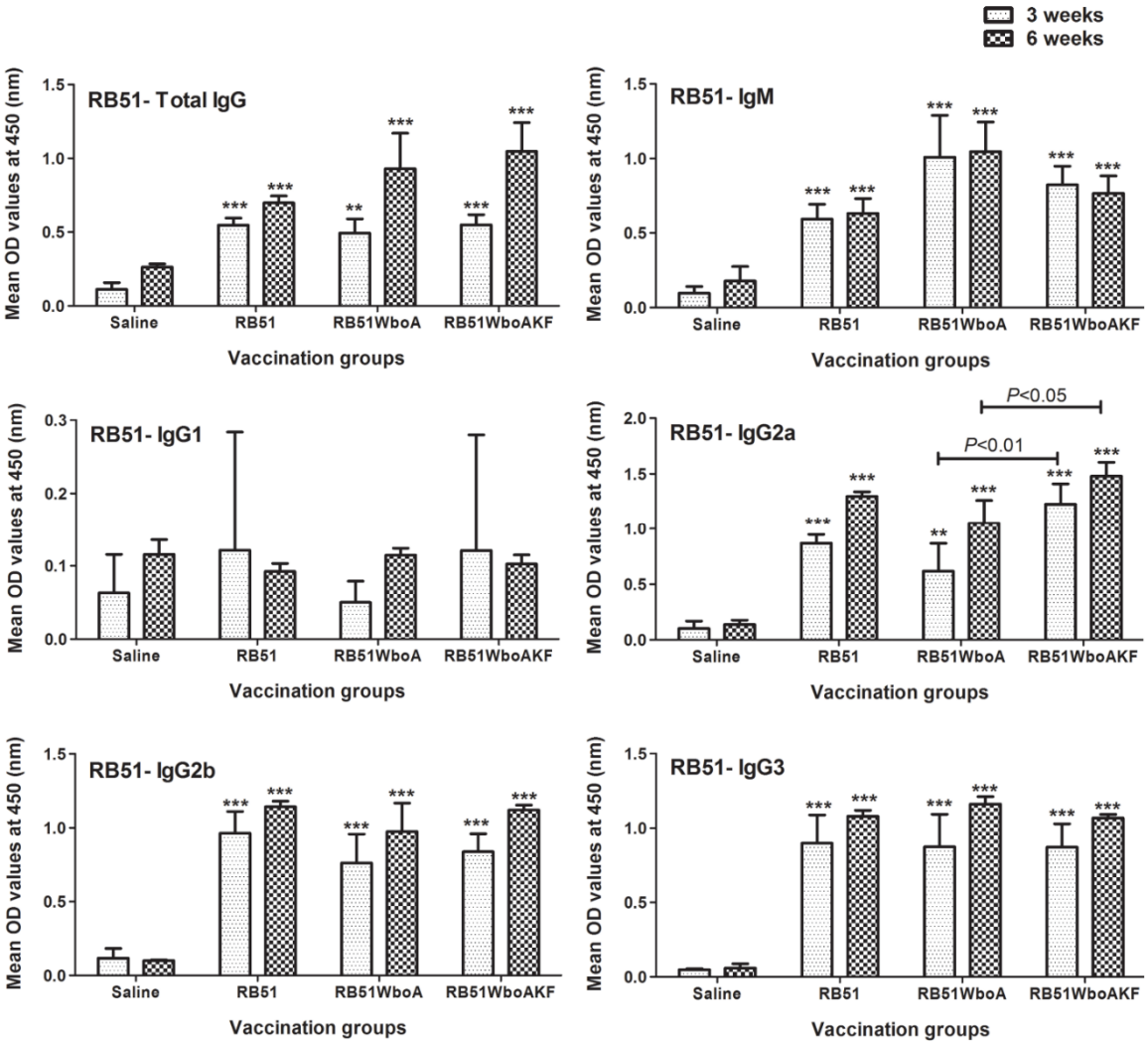

Figure 5.8. Detection of RB51-specific antibodies in the serum of mice vaccinated with live recombinant RB51WboAKF. Mice were vaccinated with live RB51, RB51WboA, RB51WboAKF, or inoculated with saline. Serum samples were collected at 3 and 6 weeks after vaccination, were diluted 1 in 200 and assayed for the presence of RB51-specific IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies by indirect ELISA. Results are shown as mean ± standard deviation (n=4) of absorbance of the color developed. Asterisks indicate statistically significant differences from the corresponding saline group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. OD, optical density.

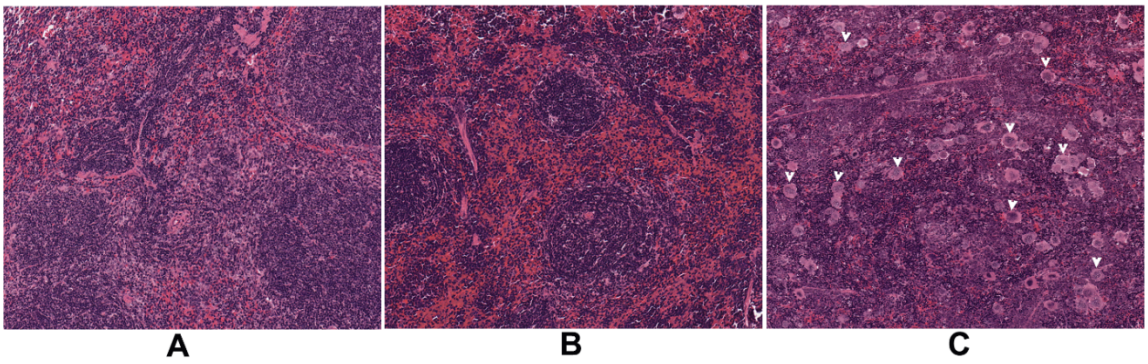

Figure 5.9. Spleen histology in BALB/c (A) saline-inoculated mice and mice infected intra-peritoneally with 10^8 CFU-equivalent of live (B) RB51 and (C) RB51WboAKF. The mice were sacrificed 7 days post-vaccination and a portion of the spleen was fixed in formalin, processed and stained with hematoxylin and eosin. Pictures were taken with a 10X magnification. White arrowheads indicate megakaryocytes, a key feature of extramedullary hematopoiesis.

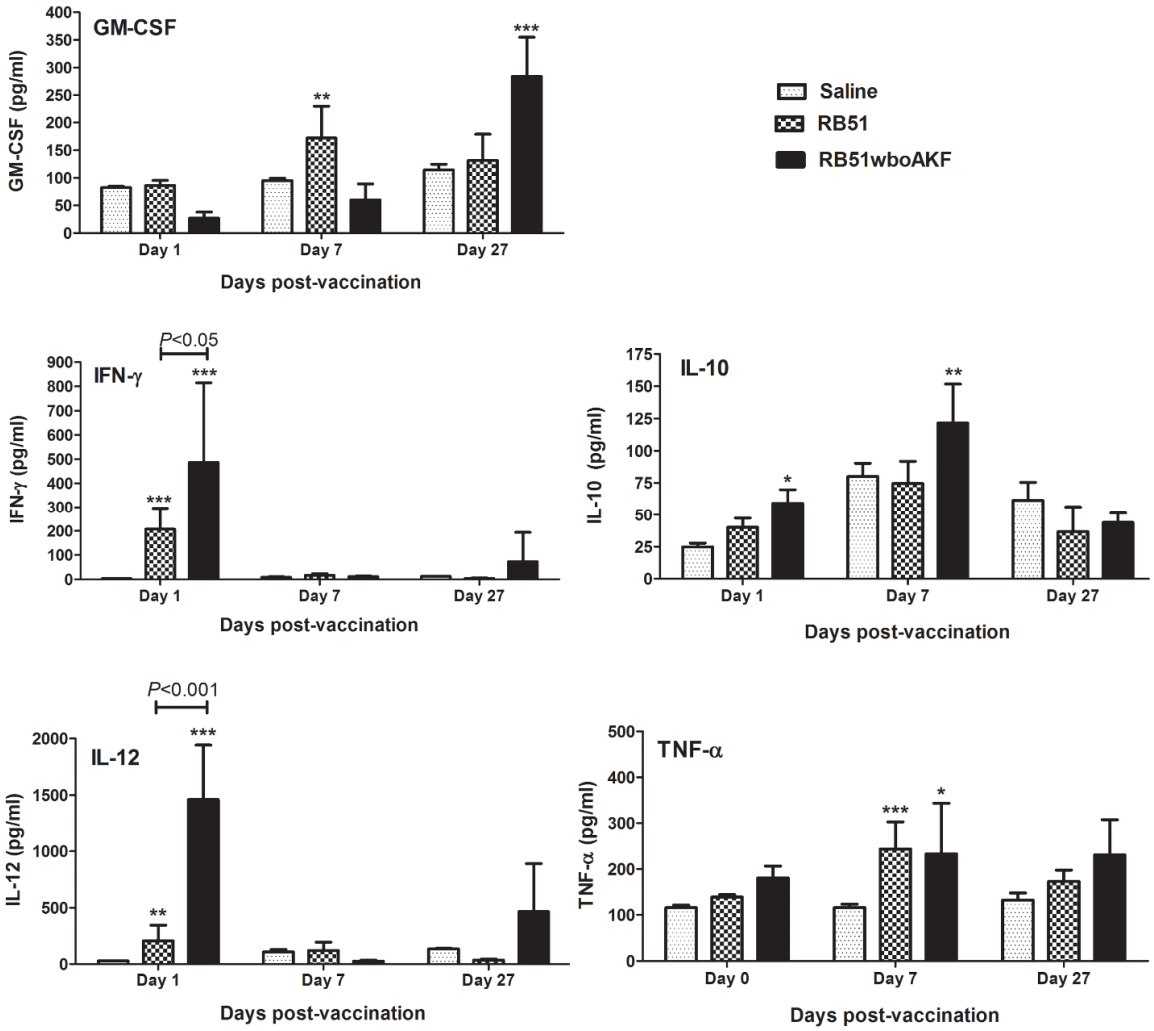

Figure 5.10. Detection of specific cytokines in the serum of RB51WboAKF vaccinated mice. Mice were immunized intra-peritoneally with 10^8 CFU-equivalent of live RB51, RB51WboAKF, or inoculated with saline. Values are shown as mean ± standard deviation (n=4). Asterisks indicate statistically significant differences from the corresponding saline group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

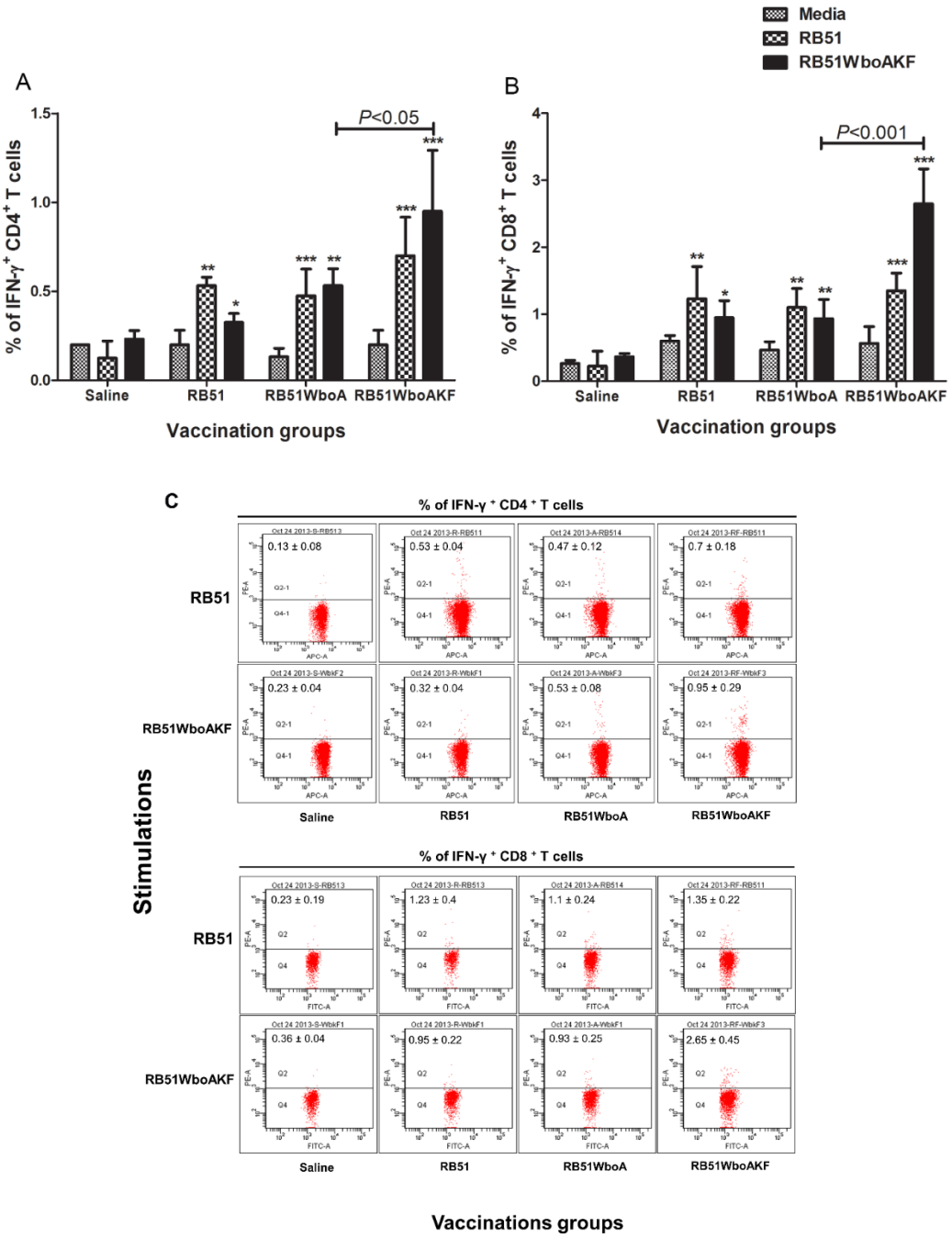

Figure 5.11. Flow cytometric analysis showing the percentage of interferon-γ secreting (A) $CD4^+$ and (B) $CD8^+$ T cells in the spleens of mice immunized with live recombinant RB51WboAKF. Mice were immunized intra-peritoneally with 10^8 CFU-equivalent of live

RB51, RB51WboA, RB51WboAKF, or inoculated with saline. The mice splenocytes were stimulated with media (unstimulated), heat-killed strains RB51 and RB51WboAKF and the percentages of IFN-γ secreting (A) $CD4^+$ and (B) $CD8^+$ T cells were analyzed by flow cytometry. (C) Representative figure of the flow cytometric analysis. Values are shown as mean ± standard deviation (n=4). Asterisks indicate statistically significant differences from the corresponding unstimulated control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

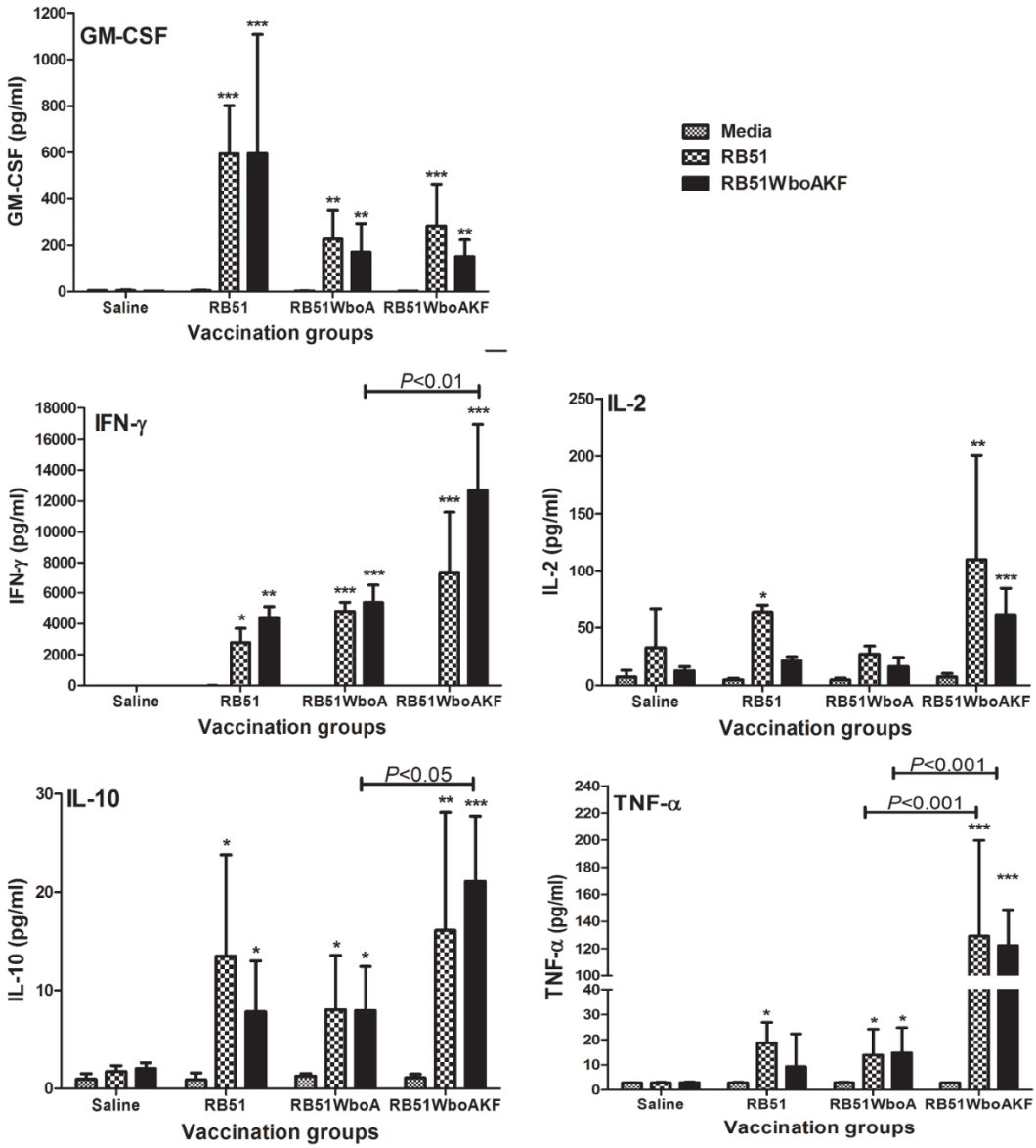

Figure 5.12. Detection of specific cytokines produced by splenocytes of mice vaccinated with live recombinant RB51WboAKF. Mice were vaccinated intra-peritoneally with 10^8 CFU-equivalent of live RB51, RB51WboA, RB51WboAKF and saline-inoculated mice after in vitro stimulation with media (unstimulated), heat-killed RB51 and RB51WboAKF. Values are shown as mean ± standard deviation (n=4). Asterisks indicate statistically significant differences from the corresponding unstimulated control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

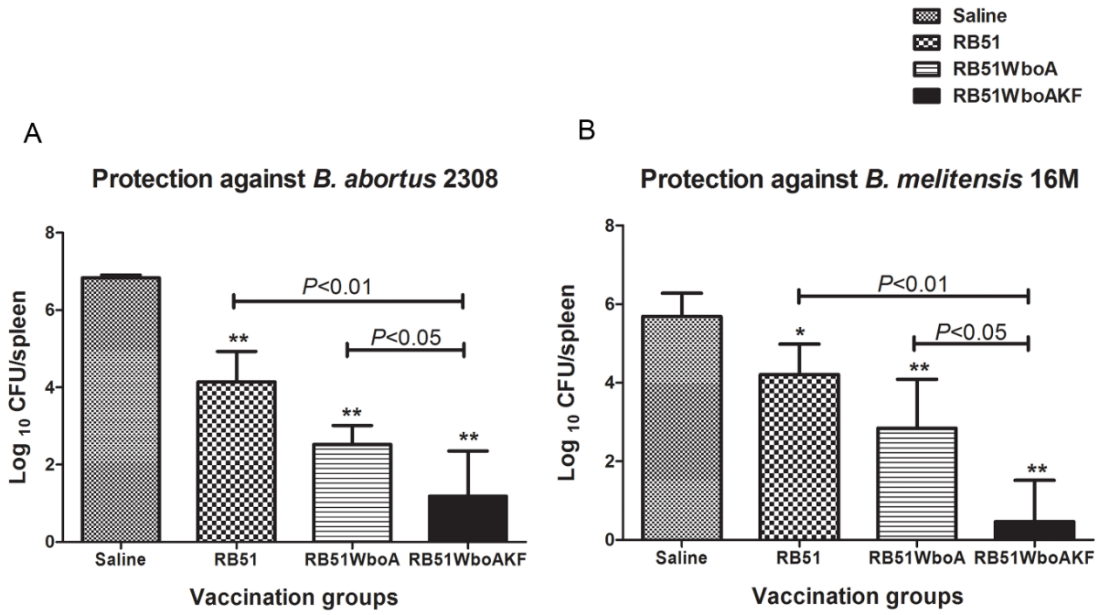

Figure 5.13. Determination of the protective efficacy of strain RB51WboAKF against virulent strains *B. abortus* 2308 and *B. melitensis* 16M challenge. Mice vaccinated intra-peritoneally with live RB51, RB51WboA, RB51WboAKF and saline-inoculated mice were challenged by i.p. inoculation of 3×10^4 CFU of virulent strains (A) *B. abortus* 2308 and (B) *B. melitensis* 16M. 2 weeks post-challenge, the mice were euthanized and the *Brucella* CFU in their spleens were determined. Results are shown as mean ± standard deviation (n=5) of the log CFU of *Brucella* recovered from spleens. Asterisks indicate statistically significant differences from the corresponding saline group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

CHAPTER 6. ORAL IMMUNIZATION OF MICE WITH GAMMA-IRRADIATED *BRUCELLA NEOTOMAE* INDUCES PROTECTION AGAINST INTRAPERITONEAL AND INTRANASAL CHALLENGE WITH VIRULENT *BRUCELLA ABORTUS* 2308

Abstract

Brucella spp. are Gram-negative, facultative intracellular coccobacilli that cause one of the most frequently encountered zoonosis worldwide. Humans naturally acquire infection through consumption of contaminated dairy and meat products and through direct exposure to aborted animal tissues and fluids. No vaccine against brucellosis is available for use in humans. In this study, we tested the ability of orally inoculated gamma-irradiated *B. neotomae* and *B. abortus* RB51 in a prime-boost immunization approach to induce antigen-specific humoral and cell mediated immunity and protection against challenge with virulent *B. abortus* 2308. Heterologous prime-boost vaccination with *B. abortus* RB51 and *B. neotomae* and homologous prime-boost vaccination of mice with *B. neotomae* led to the production of serum and mucosal antibodies specific to the smooth LPS. The elicited serum antibodies included the isotypes of IgM, IgG1, IgG2a, IgG2b and IgG3. All oral vaccination regimens induced antigen-specific $CD4^+$ and $CD8^+$ T cells capable of secreting IFN-γ and TNF-α. Upon intra-peritoneal challenge, mice vaccinated with *B. neotomae* showed the highest level of resistance against virulent *B. abortus* 2308 colonization in spleen and liver. Experiments with different doses of *B. neotomae* showed that all tested doses of 10^9, 10^{10} and 10^{11} CFU-equivalent conferred significant protection against the intra-peritoneal challenge. However, a dose of 10^{11} CFU-equivalent of *B. neotomae* was

required for affording protection against intranasal challenge as shown by the reduced bacterial colonization in spleens and lungs. Taken together, these results demonstrate the feasibility of using gamma-irradiated *B. neotomae* as an effective and safe oral vaccine to induce protection against respiratory and systemic infections with virulent *Brucella*.

Introduction

Brucella species, Gram-negative, facultative intracellular coccobacilli, are the causative agents of brucellosis, a chronic bacterial infection in a variety of mammals, including humans. *B. melitensis, B. abortus* and *B. suis* are the most frequent causes of human infections [1]. These *Brucella* species are also categorized in the class B list of select agents by the CDC due to their highly infectious nature and their potential use in bio-warfare. In natural animal hosts and wildlife reservoirs, brucellosis commonly results in abortion and infertility. Chronically infected animals also shed the bacteria in milk. Human brucellosis is truly a zoonotic disease. Humans usually get infected by consuming unpasteurized contaminated milk or dairy products, or by getting exposed to infected animal tissues or secretions. Few human infections are also documented to occur through accidental exposure to live bacteria in the laboratory or inoculation of live vaccine strains used for controlling animal brucellosis in the field. Human brucellosis can manifest in a variety of clinical symptoms, starting from a subclinical infection to a protracted febrile illness which can progress to lethal endocarditis [1,2]. Treatment of brucellosis requires prolonged therapy with a mixture of antibiotics; even then relapses of infection are often noticed. There is no vaccine available for use in humans against brucellosis.

A safe and effective human vaccine would benefit as a prophylactic measure to protect personnel at high risk of occupational exposure to pathogenic *Brucella*, especially in *Brucella* endemic regions. Cell-mediated immunity plays a major role in enhancing the resistance against brucellosis in animals. Antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes that secrete Th1-type cytokines such as IFN-γ and TNF-α are important in immunity against *Brucella* infection [3-5]. In some animal species, antibodies to the O-polysaccharide (O-PS) of the lipopolysaccharide also play a role in protection against

infections by *B. abortus*, *B. melitensis* and *B. suis* [6-8]. Only live vaccines are shown to be effective in providing long term protection against brucellosis in animals. Attenuated strains such as *B. abortus* S19 and RB51, and *B. melitensis* Rev1 are used as parenteral vaccines to immunize cattle, and sheep and goats, respectively. None of the live vaccines licensed for use in domestic animals are considered safe for human application [9,10].

Gamma-irradiated bacteria cannot replicate but remain metabolically active, and they can be a safer alternative to live bacteria for immunization purposes. We previously showed that parenteral immunization of mice with gamma-irradiated *B. abortus* RB51 or *B. neotomae* induces protection against challenge with virulent *Brucella* spp. [11,12]. As parenteral route of vaccination is seldom favored due to its invasive delivery and is unlikely to be a preferred route of immunization against brucellosis in humans, in this study we examined the feasibility of oral vaccination with gamma-irradiated *B. neotomae* and *B. abortus* RB51 in a prime-boost approach to induce protection against systemic and mucosal challenge infections.

We show that oral immunization with gamma-irradiated *B. neotomae* in a homologous prime-boost regimen results in production of antigen-specific antibody, cell-mediated and mucosal immune responses and increased resistance to intra-peritoneal and intranasal challenge with *B. abortus* 2308.

Materials and methods

Bacterial strains

B. neotomae strain 5K33 was obtained from the American Type Culture Collection, Manassas, VA. *B. abortus* vaccine strain RB51 and *B. abortus* virulent strain 2308 were from our culture collection. *B. neotomae* and RB51 were grown in tryptic soy broth (TSB) or tryptic soy agar (TSA) at 37°C. All experiments with *B. neotomae* were performed in a Biosafety level (BSL)-2 facility using BSL-3 practices. All experiments with virulent *B. abortus* were performed in a BSL-3 facility approved for the select agents work.

Vaccine preparation

B. neotomae and *B. abortus* RB51 were grown in TSB to mid log phase, and aliquots containing approximately 1×10^{12} colony forming units (CFU)/ml were exposed to 350 krads of gamma irradiation using a ^{60}Co source irradiator (Gammacell 220 irradiator). The irradiated aliquots were plated on TSA to confirm the inability of the bacteria to replicate. The irradiated bacteria were stored at 4ºC until use.

Quantification of irradiated B. neotomae in mice spleens and mesenteric lymph nodes by real-time PCR

A group of 8 female BALB/c mice were orally administered with 1×10^{11} CFU-equivalent of gamma-irradiated *B. neotomae*. On days 1 and 3 post-vaccination, 4 mice from the group were euthanized and their spleens and mesenteric lymph nodes were collected aseptically. The organs were homogenized in PBS and DNA from the homogenates were extracted using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen Inc.). Quantification of *B. neotomae* DNA in the samples was accomplished using real-time PCR as previously described [11].

Immunization of mice

Groups of 4 female BALB/c mice of 4 to 6 weeks of age were used for the study. Mice were purchased from a commercial source (Harlan Laboratories, USA), and housed in cages with microisolator tops at 4 mice per cage. Feed and water were provided ad libitum. Housing conditions included standard 12 hrs light/dark cycle, controlled humidity (55%) and room temperature (22ºC). After 1 week of acclimatization, mice were administered with vaccine or control formulation.

The gamma-irradiated bacteria were pelleted, washed with sterile phosphate-buffered saline (PBS) and adjusted to 5×10^{11} CFU-equivalent/ml. In some designated experiments, the irradiated bacteria were further diluted to concentrations of 5×10^{10} cells/ml and 5×10^{9}

cells/ml. A total of 200 µl of the vaccine suspension was used to vaccinate mice 15 mins after oral administration of 100 µl of sterile 10% sodium bicarbonate via a disposable feeding needle attached to a 1-ml syringe.

Comparison of different prime-boost immunization strategies

Mice were vaccinated by oral inoculation of 1×10^{11} CFU-equivalent of gamma-irradiated RB51 and/or gamma-irradiated *B. neotomae* in a prime-boost immunization regimen as shown in Table 6.1. Priming dose was administered at day 0. Booster immunizations with the same dose as the priming dose were given on days 3, 7 and day 10. A group of mice inoculated with saline was used as a negative control.

Comparison of different vaccine doses of the *B. neotomae* vaccine

Mice were vaccinated by three oral inoculations, on days 0, 3 and 10, with either 1×10^{9} CFU-equivalent or 1×10^{10} CFU-equivalent or 1×10^{11} CFU-equivalent of gamma-irradiated *B. neotomae*. A group of mice inoculated with saline was used as a negative control.

Collection of blood and intestinal secretions

Blood was collected by puncturing the retro-orbital plexus of the anesthetized mice at one and two weeks post-immunization (p.i) (1 and 2 weeks after the last booster immunization, respectively). The serum was separated and used for detection of antigen-specific antibodies by enzyme-linked immunosorbent assay (ELISA). At 2 weeks p.i., CO_2 asphyxiation followed by cervical dislocation was used to euthanize all the mice. The small intestines were separated and the intestinal contents were collected by flushing with 3 ml of PBS and centrifuged to remove the particulate matter. The supernatants were transferred to clean tubes and were used for the analysis of antigen-specific antibody responses by ELISA. Spleens of the euthanized mice were collected aseptically and used for analysis of the antigen-specific cell-mediated immune responses by flow-cytometry.

Preparation of B. neotomae LPS antigen

Butanol-water extraction was used to obtain total LPS from live bacteria as previously described [13,14]. Briefly, 10g of wet pellet obtained by centrifugation of live *B. neotomae* organisms was resuspended in PBS at a concentration of 0.25 g wet weight/ml followed by addition of an equal volume of water saturated butanol. The aqueous phase was collected by centrifugation at 35,000×g for 15 mins. 4 volumes of cold methanol was added to precipitate the LPS. The precipitate was dissolved in 0.1M Tris buffer (pH 8) containing 2% SDS and 2% mercaptoethanol and heated for 5 mins at 100ºC. Following another incubation with proteinase K for 3 hrs at 60ºC, cold methanol was added to precipitate LPS. The precipitate was dissolved in water. SDS-PAGE of the polyacrylamide gel was carried out to determine the purity of the LPS.

Indirect ELISA

Levels of *B. neotomae* LPS-specific immunoglobulin G (total IgG), as well as selected IgG isotypes and IgM were determined by indirect ELISA [15]. Level of serum IgG with specificity to heat-killed RB51 total antigens was also determined. In the intestinal secretions, levels of IgG, IgM and IgA with specificity to *B. neotomae* LPS and RB51 total antigens were determined. Using carbonate buffer, pH 9.6, *B. neotomae* LPS was diluted 1 in 10, and the RB51 total antigens were diluted to obtain 1×10^9 CFU-equivalent/ml. The diluted antigens were used for coating wells (100 µl/well) of polystyrene plates (Nunc-Immunoplate with maxisorp surface). The plates were incubated at 4°C for 12 hrs. They were subsequently washed with wash buffer (Tris-buffered saline (TBS), pH 7.4, with 0.05% Tween 20) and blocked with 5% skim milk in TBS. Following an incubation of 1 hr at 37°C, mouse sera with a 1:200 dilution in blocking buffer were added to the wells (50 µl/well). All serum samples were tested in duplicates. For intestinal secretions, 1 in 10 dilution was used. The plates were incubated for 4 hrs. Appropriately diluted mouse-specific horseradish peroxidase-labeled immunoglobulin conjugates (Southern Biotechnology Associates Inc, Birmingham, Alabama) were added (50 µl/well) to the wells

after washing the plate four times with wash buffer. After another hr of incubation at room temperature, the plates were washed four times and 100 µl of substrate solution (TMB Microwell peroxidase substrate; KPL, Gaithersburg, MD) was added to each well. 100 µl of stop solution (0.185 M sulfuric acid) was further added to the wells in order to stop the enzymatic reaction, and the absorbance (450nm) was recorded using a microplate reader (Molecular devices, Sunnyvale, CA).

Intracellular cytokine staining and flow cytometry analysis

Intracellular staining for IFN-γ and TNF-α was performed as previously described with some modifications [16]. Spleens were collected aseptically after euthanizing the mice and single cell suspensions of the splenocytes were prepared. ACK lysis buffer was used to lyse the RBCs and the splenocytes (10^6 per well) were seeded in 96 well flat-bottomed plates. They were cultured with different stimulants: 10^7 CFU-equiv. of gamma-irradiated *B. neotomae* and 10^7 CFU-equiv. of gamma-irradiated RB51. As controls, cells were stimulated with plain medium and 2.5 µg/ml of concanavalin (ConA). They were incubated in a humidified incubator with 5% CO_2 for 8 hrs at 37 °C. Subsequently, brefeldin A (Golgistop; Pharmingen) was added to the splenocytes and the plates were incubated for another 8 hrs. Cells from each well were resuspended in PBS containing 1% BSA and 0.2% sodium azide (FACS buffer) and were incubated with PE-Cy7-conjugated anti-mouse CD8 antibody (BD Pharmingen, clone53-6.7) and APC-conjugated anti-mouse CD4 antibody (BD Pharmingen, clone L3T4-RM 4.5) for surface staining. They were washed three times with FACS buffer and were further stained for intracellular IFN-γ and TNF-α by incubating with PE-conjugated rat anti-mouse IFN-γ antibody and FITC-conjugated rat anti-mouse TNF-α antibody using the Cytofix/Cytoperm kit (Pharmingen). Cells stained with PE-conjugated rat IgG1 and FITC-conjugated rat IgG1 antibody served as the isotype controls. BD FACS Canto II™ Flow cytometer (BD Biosciences, CA, USA) was used to acquire the data which was analyzed using BD FACSDIVA version 6 software (BD Biosciences, CA, USA) and the proportion of $CD4^+$and $CD8^+$ T cells that secreted IFN-γ and TNF-α were determined.

Protection experiments

Protection experiments were performed at Virginia Tech in an ABSL-3 facility that was approved for work with select agents. Female BALB/c mice of 4-6 weeks of age (Harlan Laboratories, USA) were used for the studies. Mice were housed in individually ventilated cages with high-efficiency particulate arresting-filtered air. Feed and water were provided ad libitum. Housing conditions included standard 12 hrs light/dark cycle, controlled humidity (55%) and room temperature (22^{o}C). After 1 week of acclimatization, mice were administered with vaccine or control formulation.

Analysis of protective efficacy of the different prime-boost immunization regimens

Groups of 5 mice were vaccinated by oral inoculation of 1×10^{11} CFU-equiv. of gamma-irradiated RB51 and/or gamma-irradiated *B. neotomae* in a prime-boost immunization regimen; immunizations were performed (as as per *Immunization of mice* section: comparison of different prime-boost immunization strategies). A group (n=5) of mice inoculated with saline alone served as control. Two weeks p.i, each mouse was challenged by i.p. inoculation with 1×10^{4} CFU of *B. abortus* 2308. Two weeks post challenge, the mice were euthanized and the number of bacterial CFUs in their spleens, lungs and livers were enumerated as previously described [17,18]. Briefly, two weeks post challenge, the mice were euthanized and their spleens, lungs and livers were collected aseptically. Organs were suspended in 1 ml of sterile PBS and were separately homogenized using tissue grinders. Ten-fold serial dilutions of the tissue homogenates were prepared in saline, and 50 µl of each dilution was plated on TSA. After 3-5 days of incubation at 37 °C, the *Brucella* CFUs were counted and the bacterial burden per organ was calculated.

Analysis of the protective efficacy of different vaccine doses of *B. neotomae*

Groups of 10 mice were vaccinated by three oral inoculations on days 0, 3 and 10 with either 1×10^{9} CFU-equiv. or 1×10^{10} CFU-equiv. or 1×10^{11} CFU-equiv. of gamma-irradiated

B. neotomae (as per *Immunization of mice* section: comparison of different vaccine doses of the *B. neotomae* vaccine). A group (n=10) of mice inoculated with saline alone served as control. Two weeks p.i. (2 weeks after the inoculation of the last booster dose), five mice from each group were challenged by i.p. inoculation with 5×10^4 CFU of *B. abortus* 2308, and the remaining five mice were challenged by i.n. inoculation with 4×10^6 CFU of *B. abortus* 2308. Two weeks post challenge, the mice were euthanized and the bacterial burden per organ was calculated.

Statistical analyses

Absorbance values of ELISA and flow cytometry data were analyzed for differences among different vaccinated groups by performing analysis of variance with post hoc Tukey for pair-wise comparison using SPSS version 21.0 (SPSS Inc., an IBM company, USA). Following bacterial challenge experiments, student t-test modified for unequal variances between groups was carried out to compare the log transformed CFU values in organs obtained from each vaccinated group of mice with the respective saline group. *P* values of <0.05 were considered significant.

Ethics statement

The protocols of the mice experiments conducted in this study were approved by the Institutional Animal Care and Use Committees at Purdue University (Approval # 1112000488) and Virginia Tech (Approval # CVM-10-048). The animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Blood was collected from the retro-orbital plexus from mice under anesthesia. A commercially available rodent anesthesia machine that uses oxygen and an isoflurane precision-vaporizer for supplying regulated concentration of anesthetic mixture (Vetamac, Inc., Rossville, Indiana) was used for anesthetizing mice. To reduce pain following the bleeding, a drop of proparacaine hydrochloride ophthalmic solution (Bausch & Lomb, Tampa, Florida) was placed on the

eye. Mice infected with virulent *Brucella abortus* do not develop any clinical disease or show signs of suffering for the duration of the experiments conducted in this study. Therefore, no humane endpoints were utilized for the mice in this study.

Results

Persistence of gamma-irradiated B. neotomae in mice spleens and mesenteric lymph nodes following oral immunization

The translocation of orally administered gamma-irradiated *B. neotomae* from gut lumen to mesenteric lymph node (MLN) and spleen was determined by extracting DNA from tissues and performing a quantitative real-time PCR specific to *Brucella*. Following oral inoculation of 10^{11} CFU-equivalent of gamma-irradiated *B. neotomae*, the bacterial DNA could be detected in mesenteric lymph nodes and in spleens by day 1 and persisted there for at least three days (Fig. 6.1).

Induction of antigen-specific antibody immune responses following oral prime-boost immunization with gamma-irradiated B. neotomae and/or gamma-irradiated B. abortus RB51

Levels of antibodies specific to *B. neotomae*-smooth LPS and RB51 total antigens in serum samples collected at 1 and 2 weeks p.i. (1 and 2 weeks after the inoculation of the last booster dose, respectively) were determined by indirect ELISA. Immunization of mice with IRBn, IRBn/RB51 and IRRB51/Bn resulted in the induction of significantly higher levels of serum IgG and IgM antibodies specific to LPS when compared with the saline inoculated mice and the group of mice vaccinated with IRRB51 (Fig. 6.2A). However, there was no significant differences among the three vaccination groups (Fig. 6.2A). Significantly higher levels of LPS-specific IgG2a, IgG2b and IgG3 were present in the serum samples of mice immunized with IRBn and IRBn/RB51 at 1 week and 2 week p.i.

(Fig. 6.2A). Also, mice vaccinated with IRBn and IRBn/RB51 had higher levels of LPS-specific IgG1 antibodies at 2 weeks p.i.; however, only mice vaccinated with IRBn had significantly increased levels of serum IgG1 antibodies specific to LPS at 1 week p.i. (Fig. 6.2A). In contrast, vaccination of mice with IRRB51/Bn did not lead to the induction of significant levels of LPS-specific IgG1 antibodies (Fig. 6.2A). Furthermore, significantly higher levels of LPS-specific IgG2a, IgG2b and IgG3 antibodies were detected in the serum of mice vaccinated with IRRB51/Bn only at 2-weeks p.i. (Fig. 6.2A).

Oral immunization of mice with IRBn, IRRB51/Bn and IRBn/RB51 led to the induction of significantly higher levels of LPS-specific IgG antibodies in the intestinal secretions when compared with the saline inoculated control and the IRRB51 vaccinated group (Fig. 6.3A). Only vaccination of mice with IRBn resulted in the induction of significantly increased levels of LPS-specific IgM and IgA antibodies in the intestinal secretions when compared to the other vaccinated groups (Fig. 6.3A).

All four vaccinated groups of mice had significantly higher levels of IgG serum antibodies specific to RB51 total antigens (Fig. 6.2B), as well as significantly higher levels of RB51-specific IgG, IgM and IgA antibodies in the intestinal secretions (Fig. 6.3B) than the saline inoculated control mice. There was no difference in the levels of RB51-specific antibodies among the different groups (Fig. 6.2B and 6.3B).

Induction of antigen-specific cell-mediated immune responses following oral prime-boost immunization with gamma-irradiated B. neotomae and/or gamma-irradiated B. abortus RB51

Significantly higher proportions of antigen-specific IFN-γ-and TNF-α-secreting $CD4^+$ and $CD8^+$ T cells were detected in all vaccinated groups when compared to the respective unstimulated controls and the saline inoculated control group (Fig. 6.4A and B). Stimulation of the splenocytes with irradiated RB51 resulted in the induction of significantly increased proportions of RB51-specific $CD4^+$ T cells secreting IFN-γ and TNF-α in mice immunized with IRRB51 than the other vaccinated groups (Fig. 6.4A and B). There was no significant difference in the proportion of IFN-γ and TNF-α secreting

$CD8^+$ T cells specific to RB51 and those that were specific to *B. neotomae* among the different vaccinated groups (Fig. 6.4A and B).

Protective efficacy against intra-peritoneal challenge with virulent B. abortus 2308 following oral prime-boost immunization with gamma-irradiated B. neotomae and/or gamma-irradiated B. abortus RB51

Immunization with IRBn and IRBn/RB51 resulted in a significant reduction in the number of bacterial CFUs of the virulent *Brucella* in the spleen after i.p. challenge with virulent *B. abortus 2308* (Table. 6.2). In contrast, the bacterial burden in the spleen of mice immunized with IRRB51 was not significantly different from that of the saline inoculated control group (Table. 6.2). All three vaccinations resulted in reduced bacterial load of virulent strain in livers (Table. 6.2). The bacterial loads in lungs of the vaccinated mice were not statistically different from that of the saline group (Table. 6.2).

Induction of antigen-specific antibody immune responses following oral homologous prime-boost immunization with different vaccine doses of gamma-irradiated B. neotomae

We examined the effect of different doses of gamma-irradiated *B. neotomae* vaccine on induction of specific immune responses and protection against parenteral and mucosal challenge with virulent *B. abortus*. Three different doses, 1×10^9 CFU-equiv. or 1×10^{10} CFU-equiv. or 1×10^{11} CFU-equiv. of gamma-irradiated *B. neotomae* were tested in a prime-boost immunization strategy, consisting of a priming dose and two booster doses. Significantly increased levels of LPS-specific IgG antibodies at 1 and 2 weeks p.i. and LPS-specific IgM antibodies at 1 week p.i. were detected in the serum of the three vaccinated groups when compared to the saline inoculated control group (Fig. 6.5). In addition, mice vaccinated with the 10^{11} CFU-equiv. dose developed significantly increased levels of LPS-specific IgG antibodies in the serum at 1 week p.i. when compared to the other vaccinated mice (Fig. 6.5).

Significantly higher levels of LPS-specific IgG and IgM antibodies in the intestinal secretions were present in all vaccinated mice (Fig. 6.6). Mice vaccinated with the 10^{11} CFU-equiv. dose contained the highest levels of IgG, IgM and IgA antibodies (Fig. 6.6).

Induction of antigen-specific CMI following oral homologous prime-boost immunization with different vaccine doses of gamma-irradiated B. neotomae

Upon in vitro stimulation of splenocytes with irradiated *B. neotomae* and irradiated RB51, significantly higher proportions of IFN-γ secreting $CD4^+$ and $CD8^+$ T cells were detected in all vaccinated groups (Fig. 6.7A). Stimulation of splenocytes with irradiated RB51 resulted in induction of significantly increased proportions of $CD4^+$ and $CD8^+$ T cells secreting IFN-γ in mice immunized with the 10^{11} CFU equiv. dose than the other groups (Fig. 6.7A). Significantly increased proportion of TNF-α secreting $CD4^+$ and $CD8^+$ T cells were detected upon stimulation of splenocytes of vaccinated mice with specific antigens (Fig. 6.7B).

Protective efficacy against intra-peritoneal and intra-nasal challenge with virulent B. abortus 2308 following oral homologous prime-boost immunization with different vaccine doses of gamma-irradiated B. neotomae

Upon i.p. challenge with *B. abortus* 2308, significantly reduced bacterial loads were detected in spleens and livers of all vaccinated mice (Table. 6.3); mice immunized with the 10^{11} CFU-equiv. dose showed the highest resistance with an average of 2.61 log and 2.49 log reduction in spleens and livers, respectively. Although the lung bacterial burden in all vaccinated groups was lower than that of the saline inoculated control group, the difference was not statistically significant (Table. 6.3); again, mice immunized with the 10^{11} CFU-equiv. dose showed the highest resistance in lungs with a 1.47 log difference in bacterial load compared to those of the saline control group.

Following intranasal challenge with *B. abortus* 2308, the bacterial CFUs in lungs and spleens of mice immunized with the 10^{11} CFU-equiv. dose were significantly lower than

those of mice inoculated with saline (Table. 6.4). Reduced bacterial burden was also detected in the livers of mice immunized with the 10^{11} CFU-equiv. dose, though the reduction was not statistically significant from the saline group (Table. 6.4).

Discussion

Vaccination by oral route is a desirable method to induce acquired immunity against infectious diseases in humans as well as animals. In most human brucellosis cases, the infection is acquired through mucosal routes. However, human brucellosis is a systemic disease, where the bacteria penetrate the epithelial barrier, spread to mononuclear phagocytic system, and affect different organ systems [19]. Therefore, an effective oral brucellosis vaccine must induce systemic immunity. Several research groups previously used live attenuated *Brucella* strains as oral vaccines [18,20,21]. The inherent safety risks associated with bacterial replication may preclude the use of attenuated *Brucella* strains as live vaccines for human brucellosis. Our previous research demonstrated that gamma-irradiated *B. abortus* strain RB51 and *B. neotomae* cannot replicate, but can induce protective antibody and CMI responses when used as vaccines to immunize mice by intraperitoneal route [11,12]. In this study, we asked if these vaccines can be administered by oral route to immunize mice against a challenge infection with virulent *B. abortus*. Our empirical selection to give multiple doses of the vaccine was driven by the currently recommended oral vaccination regimen for *Salmonella typhi*, the only oral bacterial vaccine in human use at present, which consists of 4 doses at 1-day intervals [22]. We first examined the effect of prime-boost strategy on induction of immune responses and protection. Contrary to our initial hypothesis, priming with *B. neotomae* and boosting with *B. abortus* RB51, or vice versa, did not significantly change the type of antibody and CMI responses. The only notable difference was that the mice primed with *B. abortus* RB51 and boosted with *B. neotomae* did not develop significant levels of IgG1 antibodies specific to smooth LPS even at 2 weeks p.i. (Fig. 6.2A). It is interesting that priming with *B. neotomae* and boosting with *B. abortus* RB51 did not dampen the overall antibody response to smooth LPS (Fig. 6.2A). In fact, mice subjected to this vaccination regimen had significantly

higher levels of smooth LPS-specific IgG2a and IgG2b antibodies at 1 week p.i., but the difference disappeared by 2 weeks p.i. (Fig. 6.2A). Majority of the surface proteins of *Brucella* spp. are highly conserved [23]. Therefore, it is not surprising that all tested vaccination regimens induced similar levels of RB51-specific antibodies in serum and intestinal secretions (Fig. 6.2B and 6.3B).

Our detection of smooth LPS- and RB51-specific antibodies in the intestinal secretions of the vaccinated mice is suggestive of induction of a mucosal immune response. However, the role of mucosal immunity in protection against brucellosis remains to be determined. A recent study demonstrated that mice orally vaccinated with live attenuated strains *B. abortus* RB51 or *B. melitensis* ΔznuA developed both systemic and mucosal Th1 as well as Th17 responses, but the Th17 responses played a role only in the *B. abortus* RB51-induced protection [21]. The important role of Th1 responses in protection against brucellosis is well-documented in the literature [4]. All the vaccines tested in our study induced antigen-specific $CD4^+$ and $CD8^+$ T cells that secrete IFN-γ or TNF-α in spleens, suggesting the generation of a systemic Th1 response.

In mouse brucellosis models, reduced bacterial load in spleens, livers or both, of the vaccinated mice compared to those of the unvaccinated animals is the generally used criterion for determining the vaccine-induced protective responses [24-26]. Based on this standard, only mice vaccinated with *B. neotomae* and IRBn/RB51, but not RB51, developed significant protection against intraperitoneal challenge with *B. abortus* 2308 (Table 6.2). This was corroborated by the reduced bacterial burden in lungs also, though the reduction was not significantly different from the unvaccinated mice. This observation suggests that oral vaccines that elicit antibodies to smooth LPS are effective in affording protection in mouse brucellosis models. Since the protection induced by the IRBn/RB51 vaccination regimen was not different from that of the IRBn vaccine, we then examined the effect of reduced dose of *B. neotomae* vaccine on induction of protection. All tested vaccine doses induced significant protection against the intraperitoneal challenge. However, only the highest dose provided significant protection against the intranasal challenge. Why the lower doses of vaccine could not provide protection against the intranasal challenge could not be answered based on our immune response analyses. There

was a trend of reduced serum and mucosal antibody levels to smooth LPS with reduced vaccine dose, which could have contributed to this difference. A thorough analysis of T cell responses in lungs following immunization and challenge may reveal important differences between the different vaccination doses. Additionally, determining the effect of different vaccine doses on the persistence of the vaccine bacteria in lymphoid organs may also yield information relevant to understanding the magnitude of immune responses induced at different mucosal sites. Our preliminary study indicates that following oral inoculation of mice with 10^{11} CFU-equivalent of gamma-irradiated *B. neotomae*, the bacterial DNA could be detected in mesenteric lymph nodes and in spleens by day 1 and persisted there for at least three days (Fig. 6.1).

In conclusion, these studies demonstrate the feasibility of using gamma-irradiated *B. neotomae* as oral vaccine to induce protection against both parenteral and mucosal challenge with virulent *Brucella* spp. This type of vaccine is a safer alternative to live vaccines for human brucellosis. Future studies should focus on formulating the vaccine to bypass the acidic conditions of human stomach, assessing the shelf-life of the vaccines and determining the duration of the vaccine-induced protective response.

References

[1] Franco, M. P., Mulder, M., Gilman, R. H., Smits, H. L., 2007. Human brucellosis. Lancet Infect Dis 12, 775-786.

[2] Ferreira, P., Gama, P., Correia, J., Nunes, L., Pipa, J., Nascimento, C., Alexandre, J. C., Henriques, P., Santos, J. O., 2008. *Brucella* endocarditis- case report and literature review. Rev Port Cardiol 27, 1309-1315.

[3] Araya, L. N., Elzer, P. H., Rowe, G. E., Enright, F. M., Winter, A. J., 1989. Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. J Immunol 143, 3330-3337.

[4] Murphy, E. A., Sathiyaseelan, J., Parent, M. A., Zou, B., Baldwin, C. L., 2001. Interferon-gamma is crucial for surviving a *Brucella* infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunology 103, 511-518.

[5] Zhan, Y. F., Liu, Z. Q., Cheers, C., 1996. Tumor necrosis factor alpha and interleukin-12 contribute to resistance to the intracellular bacterium *Brucella abortus* by different mechanisms. Infect Immun 64, 2782-2786.

[6] Vitry, M. A., Mambres, D. H., Trez, C. D., Akira, S., Ryffel, B., Letesson, J. J., Muraille, E., 2014. Humoral immunity and $CD4^+$ Th1 cells are both necessary for a fully protective immune response upon secondary infection with *Brucella melitensis*. J Immunol 192, 3740-3752.

[7] Araya, L. N., Winter, A. J., 1990. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infect Immun 58, 254-256.

[8] Ficht, T. A., Kahl-Mcdonagh, M. M., Arenas-Gamboa, A. M., Rice-Ficht, A. C., 2009. Brucellosis: the case for live, attenuated vaccines. Vaccine 27, D40-D3.

[9] Blasco, J. M., Diaz, R., 1993. *Brucella melitensis* Rev-1 vaccine as a cause of human brucellosis. Lancet 342, 805.

[10] Perkins, S. D., Smither, S. J., Atkins, H. S., 2010. Towards a *Brucella* vaccine for humans. FEMS Microbiol Rev 34, 379-394.

[11] Moustafa, D., Garg, V. K., Jain, N., Sriranganathan, N., Vemulapalli, R., 2011. Immunization of mice with gamma-irradiated *B. neotomae* and its recombinant strains induces protection against virulent *B. abortus*, *B. melitensis* and *B. suis* challenge. Vaccine 29, 784-794.

[12] Sanakkayala, N., Sokolovska, A., Gulani, J., HogenEsch, H., Sriranganathan, N., Boyle, S. M., Schurig, G. G., Vemulapalli, R., 2005. Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant *Brucella abortus* RB51. Clin Diagn Lab Immunol 12, 1429-1436.

[13] Morrison, D. C., Leive, L., 1975. Fractions of lipopolysaccharide from *Escherichia coli* O111-B4 prepared by two extraction procedures. J Biol Chem 250, 2911-2919.

[14] Phillips, M., Pugh, G. W., Deyoe, B. L., 1989. Chemical and protective properties of *Brucella* lipopolysaccharide obtained by butanol extraction. Am J Vet Res 50, 311-317.

[15] Vemulapalli, R., Duncan, A. J., Boyle, S. M., Sriranganathan, N., Toth, T. E., Schurig, G. G., 1998. Cloning and sequencing of *yajC* and *secD* homologs of *Brucella abortus* and demonstration of immune responses to YajC in mice vaccinated with *B. abortus* RB51. Infect Immun 66, 5684-5691.

[16] Murali-Krishna, K., Altman, J. D., Suresh, M., Sourdive, D. J. D., Zajac, A. J., Miller, J. D., Slansky, J., Ahmed, R., 1998. Counting antigen-specific CD8T cells: a reevaluation of bystander activation during viral infection. Immunity 8, 177-187.

[17] Schurig, G. G., Roop, R. M., Bagchi, T., Boyle, S., Buhrman, D., Sriranganathan, N., 1999. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 28, 171-188.

[18] Izadjoo, M. J., Bhattacharjee, A. K., Paranavitana, C. M., Hadfield, T. L., Hoover, D. L., 2004. Oral vaccination with *Brucella melitensis* WR201 protects mice against intranasal challenge with virulent *Brucella melitensis* 16M. Infect Immun 72, 4031-4039.

[19] Young, E. J., 1995. An overview of human brucellosis. Clin Infect Dis 21, 283-289.

[20] Pasquali, P., Rosanna, A., Pistoia, C., Petrucci, P., Ciuchini, F., 2003. *Brucella abortus* RB51 induces protection in mice orally infected with the virulent strain *B. abortus* 2308. Infect Immun 71, 2326-2330.

[21] Clapp, B., Skyberg, J. A., Yang, X., Thornburg, T., Walters, N., Pascual, D. W., 2011. Protective live oral brucellosis vaccines stimulate Th1 and Th17 cell responses. Infect Immun 79, 4165-4174.

[22] Dietrich, G., Griot-Wenk, M., Metcalfe, I. C., Lang, A. B., Viret, J. F., 2003. Experience with registered mucosal vaccines. Vaccine 21, 678-683.

[23] Cloeckaert, A., Verger, J. M., Grayon, M., Vizcaino, N., 1996. Molecular and immunological characterization of the major outer membrane proteins of *Brucella*. FEMS Microbiol Lett 145, 1-8.

[24] Gonzalez, D., Grillo, M. J., De Miguel, M. J., Ali, T., Arce-Gorvel, V., Delrue, R. M., Condee-Alvarez, R., Munoz, P., Lopez-Goni, I., Iriarte, M., Marin, C. M., Weintraub, A., Widmalm, G., Zygmunt, M., Letesson, J. J., Gorvel, J. P., Blasco, J. M., Moriyon, I., 2008. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLOS ONE 3, e2760.

[25] Montaraz, J. A., Winter, A. J., 1986. Comparison of living and nonliving vaccines for *Brucella abortus* in BALB/c mice. Infect Immun 53, 245-251.

[26] Kahl-McDonagh, M. M., Ficht, T. A., 2006. Evaluation of protection afforded by *Brucella abortus* and *Brucella melitensis* unmarked deletion mutants exhibiting different rates of clearance in BALB/c mice. Infect Immun 74, 4048-4057.

Table 6.1. Immunization schedule.

Vaccine group designation	Prime (1×10^{11} CFU) Day 0	Boost (1×10^{11} CFU) Day 3, 7 and 10
IRRB51	Irradiated RB51	Irradiated RB51
IRBn	Irradiated *B. neotomae*	Irradiated *B. neotomae*
IRRB51/Bn	Irradiated RB51	Irradiated *B. neotomae*
IRBn/RB51	Irradiated *B. neotomae*	Irradiated RB51

Table 6.2. Protection against intra-peritoneal challenge with virulent *B. abortus* 2308 following oral prime-boost immunization of mice with gamma-irradiated *B. neotomae* and/or gamma-irradiated *B. abortus* RB51.

	Vaccine (gamma-irradiated)	Bacterial load in log_{10}CFU (mean ± SD)	Units of protection[a]
Log_{10} CFU in spleen	None (saline)	4.66 ± 0.77	-
	RB51	4.03 ± 0.93**	0.63
	B. neotomae	2.55 ± 0.21*	2.11
	B. neotomae/RB51	2.97 ± 0.22*	1.69
Log_{10} CFU in liver	None (saline)	3.61 ± 0.11	-
	RB51	2.82 ± 0.32*	0.79
	B. neotomae	2.53 ± 0.21*	1.08
	B. neotomae/RB51	2.62 ± 0.25*	0.99
Log_{10} CFU in lung	None (saline)	2.59 ± 1.18	-
	RB51	2.71 ± 0.53	-
	B. neotomae	1.85 ± 0.46**	0.74
	B. neotomae/RB51	1.70 ± 0.28**	0.89

[a] Units of protection were calculated by subtracting the mean log_{10} CFU for a vaccinated group from the mean log_{10} CFU of the corresponding saline control group.

* Significantly different from the corresponding saline group ($P < 0.05$).

** Not significantly different from the corresponding saline group ($P > 0.05$).

Table 6.3. Protection against intra-peritoneal challenge with virulent *B. abortus* 2308 following oral homologous prime-boost immunization of mice with multiple vaccine doses of gamma-irradiated *B. neotomae*.

	Vaccine (gamma-irradiated)	Bacterial load in log_{10}CFU (mean ± SD)	Units of protection[a]
Log_{10} CFU in spleen	None (saline)	6.53 ± 0.03	-
	B. neotomae 10^9	5.27 ± 0.53*	1.26
	B. neotomae 10^{10}	5.18 ± 0.50*	1.35
	B. neotomae10^{11}	3.92 ± 1.29*	2.61
Log_{10} CFU in liver	None (saline)	4.48 ± 0.19	-
	B. neotomae 10^9	3.47 ± 0.27*	1.01
	B. neotomae 10^{10}	3.69 ± 0.61*	0.79
	B. neotomae10^{11}	1.99 ± 1.64*	2.49
Log_{10} CFU in lung	None (saline)	3.61 ± 0.88	-
	B. neotomae 10^9	3.47 ± 0.32**	0.14
	B. neotomae 10^{10}	3.16 ± 0.28**	0.45
	B. neotomae10^{11}	2.14 ± 1.15**	1.47

[a] Units of protection were calculated by subtracting the mean log_{10} CFU for a vaccinated group from the mean log_{10} CFU of the corresponding saline control group.

* Significantly different from the corresponding saline group ($P < 0.05$).

** Not significantly different from the corresponding saline group ($P > 0.05$).

Table 6.4. Protection against intra-nasal challenge with virulent *B. abortus* 2308 following oral homologous prime-boost immunization of mice with multiple vaccine doses of gamma-irradiated *B. neotomae*.

	Vaccine (gamma-irradiated)	Bacterial load in log_{10}CFU (mean ± SD)	Units of protection[a]
Log_{10} CFU in spleen	None (saline)	5.10 ± 0.61	-
	B. neotomae 10^9	5.23 ± 0.74	-
	B. neotomae 10^{10}	5.34 ± 0.39	-
	B. neotomae10^{11}	3.89 ± 0.31*	1.21
Log_{10} CFU in liver	None (saline)	3.33 ± 0.65	-
	B. neotomae 10^9	3.36 ± 0.48	-
	B. neotomae 10^{10}	3.25 ± 0.63**	0.08
	B. neotomae10^{11}	2.12 ± 1.12**	1.21
Log_{10} CFU in lung	None (saline)	5.30 ± 0.12	-
	B. neotomae 10^9	5.40 ± 0.21	-
	B. neotomae 10^{10}	4.67 ± 0.94**	0.63
	B. neotomae10^{11}	4.08 ± 0.91*	1.22

[a] Units of protection were calculated by subtracting the mean log_{10} CFU for a vaccinated group from the mean log_{10} CFU of the corresponding saline control group.

* Significantly different from the corresponding saline group ($P < 0.05$).

** Not significantly different from the corresponding saline group ($P > 0.05$).

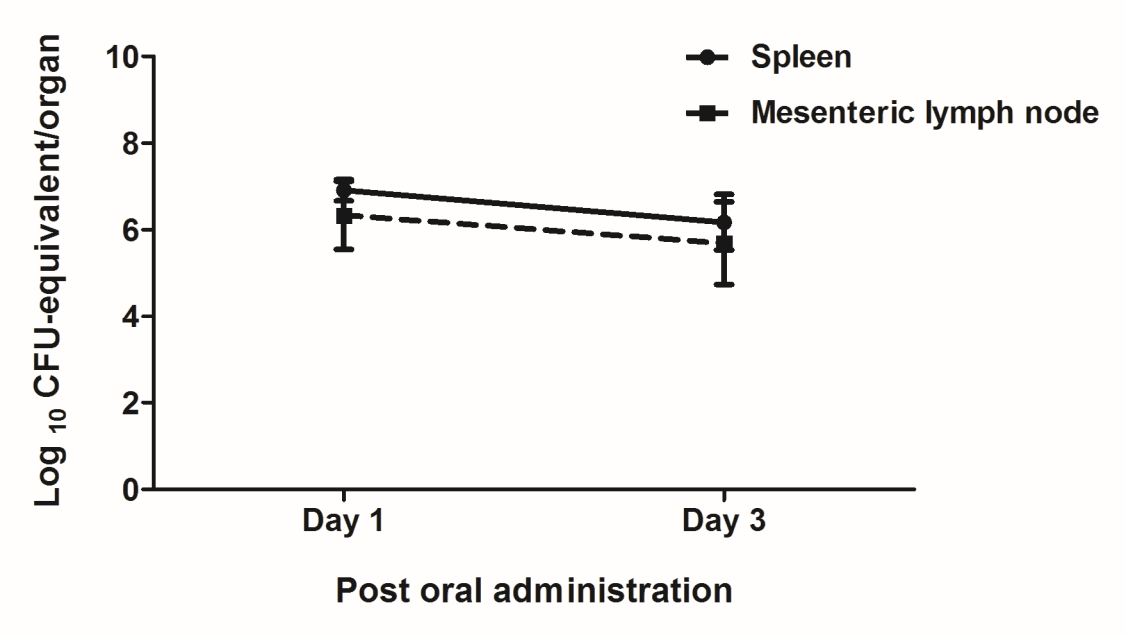

Figure 6.1. Persistence of gamma-irradiated *B. neotomae* in mouse spleens and mesenteric lymph nodes as detected by real-time quantitative PCR. A group of 8 female BALB/c mice were orally administered with 1×10^{11} CFU-equivalent of gamma-irradiated *B. neotomae*. On days 1 and 3 post-vaccination, 4 mice from the group were euthanized and their spleens and mesenteric lymph nodes were collected aseptically. The organs were homogenized in PBS and DNA from the homogenates were extracted using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen Inc.). Quantification of *B. neotomae* DNA in the samples was accomplished using real-time PCR as described in Methods and materials.

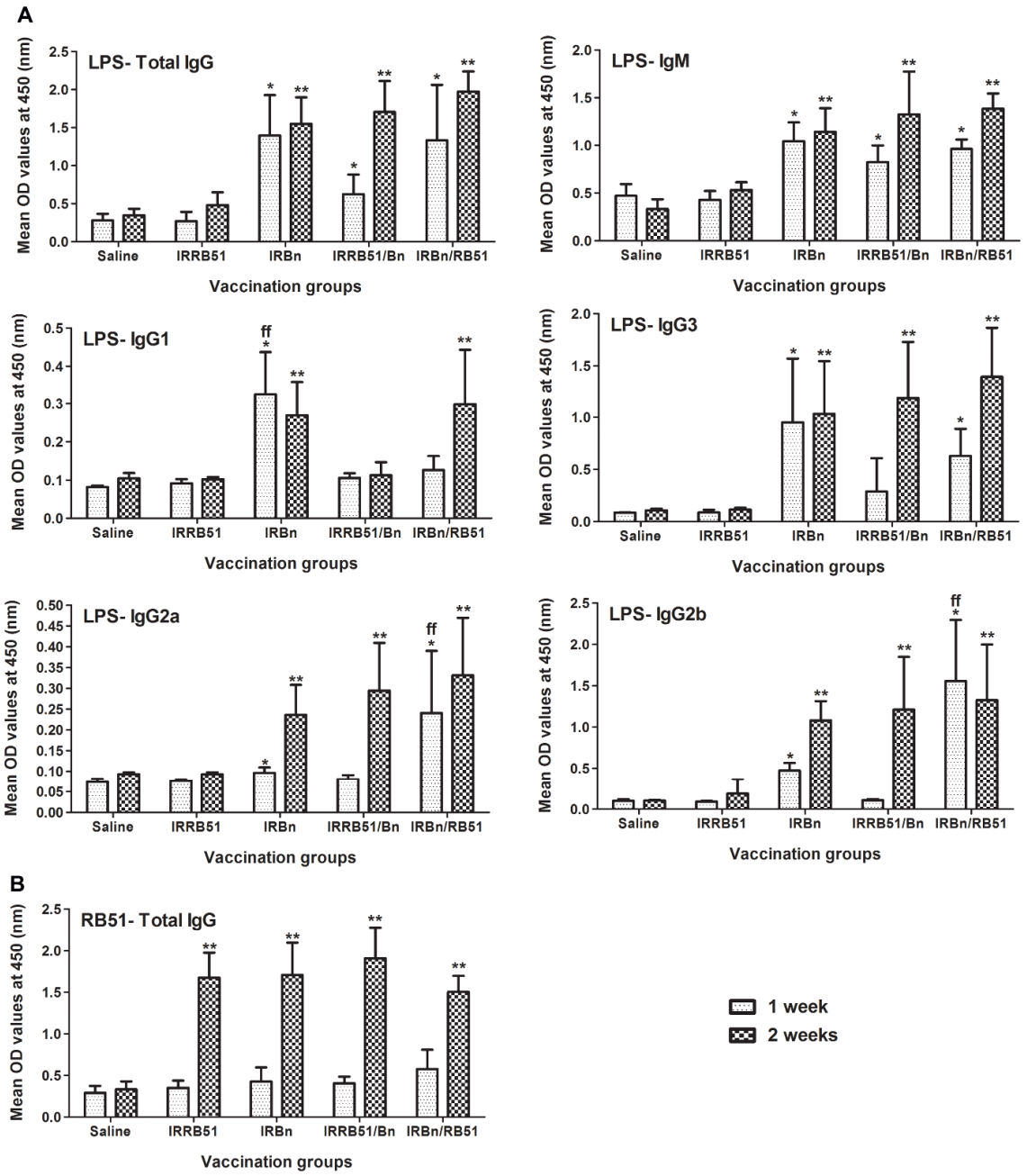

Figure 6.2. ELISA detection of (A) *B. neotomae*-specific antibodies, and (B) RB51-specific IgG antibody in serum of mice vaccinated with gamma-irradiated prime-boost regimens. Mice were vaccinated with gamma-irradiated *RB51*, *B. neotomae*, RB51/*B. neotomae* and *B. neotomae*/RB51, or inoculated with saline. Serum samples were collected at 1 and 2

weeks after the last booster vaccination, were diluted 1 in 200 and assayed for the presence of (A) LPS-specific IgG, IgM, IgG1, IgG3, IgG2a and IgG2b and (B) RB51-specific antibodies. Results are shown as mean ± standard deviation ($n = 4$) of absorbance at 450 nm of the color developed. *Significantly different from the corresponding saline group at week 1 ($P < 0.05$). **Significantly different from the corresponding saline group at week 2 ($P < 0.05$). ^{ff} Significantly different from the corresponding vaccination groups at week 1 ($P < 0.05$). OD, optical density.

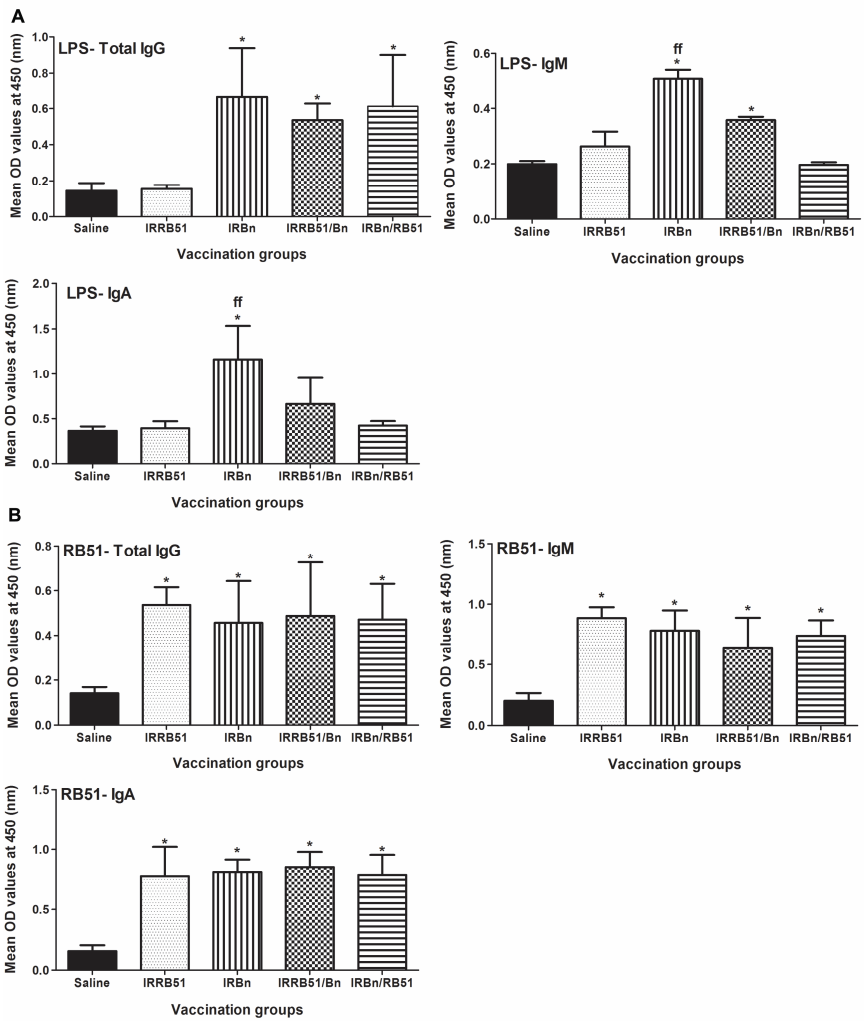

Figure 6.3. ELISA detection of IgG, IgM, IgA antibodies specific to (A) *B. neotomae* LPS, and (B) RB51 total antigens in the intestinal secretions of mice vaccinated with gamma-irradiated prime-boost regimens. Mice were vaccinated with gamma-irradiated *RB51*, *B. neotomae*, RB51/*B. neotomae* and *B. neotomae*/RB51, or inoculated with saline. Intestinal secretions were collected at 2 weeks after the last booster vaccination, were diluted 1 in 10 and assayed for the presence of (A) LPS-specific and (B) RB51-specific antibodies. Results are shown as mean ± standard deviation (n = 4) of absorbance at 450 nm of the color developed. *Significantly different from the corresponding saline group ($P < 0.05$). ^{ff} Significantly different from the corresponding vaccination groups ($P < 0.05$). OD, optical density.

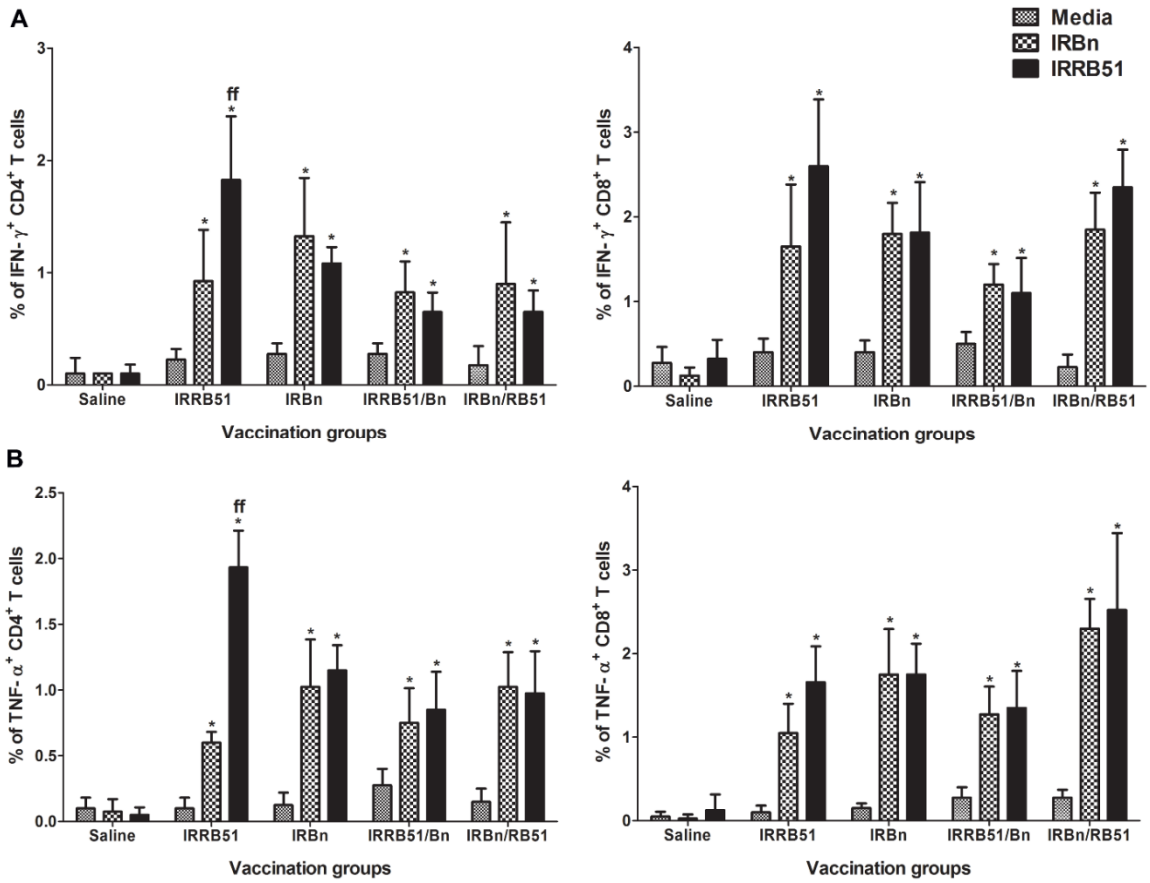

Figure 6.4. Flow cytometric analysis showing the percentage of (A) interferon-γ secreting, and (B) tumor necrosis factor-α secreting $CD4^+$ and $CD8^+$ T cells in the spleens of BALB/c mice immunized with gamma-irradiated prime-boost regimens. Mice were vaccinated with *RB51*, *B. neotomae*, RB51/*B. neotomae* and *B. neotomae*/RB51, or inoculated with saline. Splenocytes from the vaccinated mice were stimulated with media (unstimulated), gamma-irradiated *B. neotomae* and gamma-irradiated RB51 and the percentage of IFN-γ secreting and TNF-α secreting $CD4^+$ and $CD8^+$ T cells were analyzed by flow-cytometry. *Significantly different from the corresponding unstimulated control ($P < 0.05$). ^{ff} Significantly different from the corresponding vaccination groups with irradiated RB51 stimulation ($P < 0.05$).

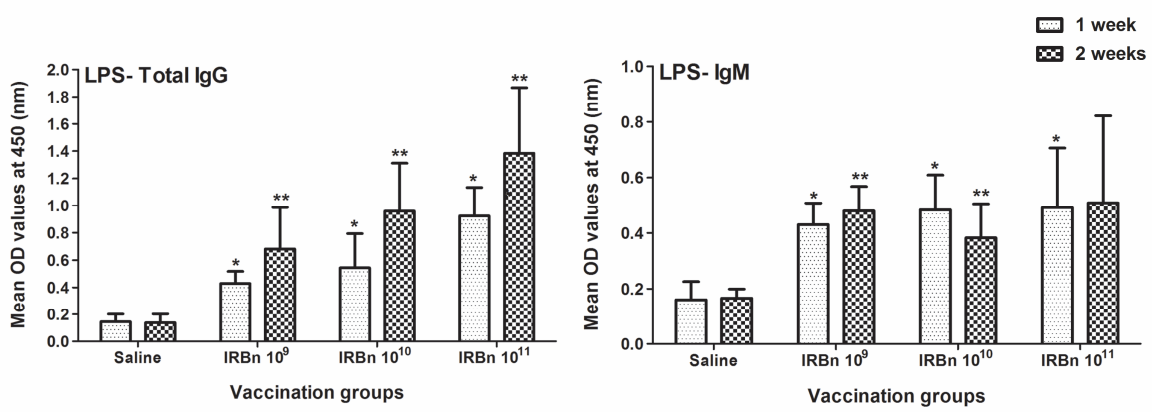

Figure 6.5. ELISA detection of IgG and IgM antibodies specific to *B. neotomae* LPS in serum of mice vaccinated with different doses of gamma-irradiated *B. neotomae*. Mice were vaccinated with 10^9 CFU-equivalent or 10^{10} CFU-equivalent or 10^{11} CFU-equivalent of gamma-irradiated *B. neotomae*, or inoculated with saline.Serum samples were collected at 1 and 2 weeks after the last booster vaccination, were diluted 1 in 200 and assayed for the presence of LPS-specific antibodies. Results are shown as mean ± standard deviation (n = 4) of absorbance at 450 nm of the color developed. *Significantly different from the corresponding saline group at week 1 ($P < 0.05$). **Significantly different from the corresponding saline group at week 2 ($P < 0.05$). ^{ff} Significantly different from the corresponding vaccination groups at week 1 ($P < 0.05$). OD, optical density.

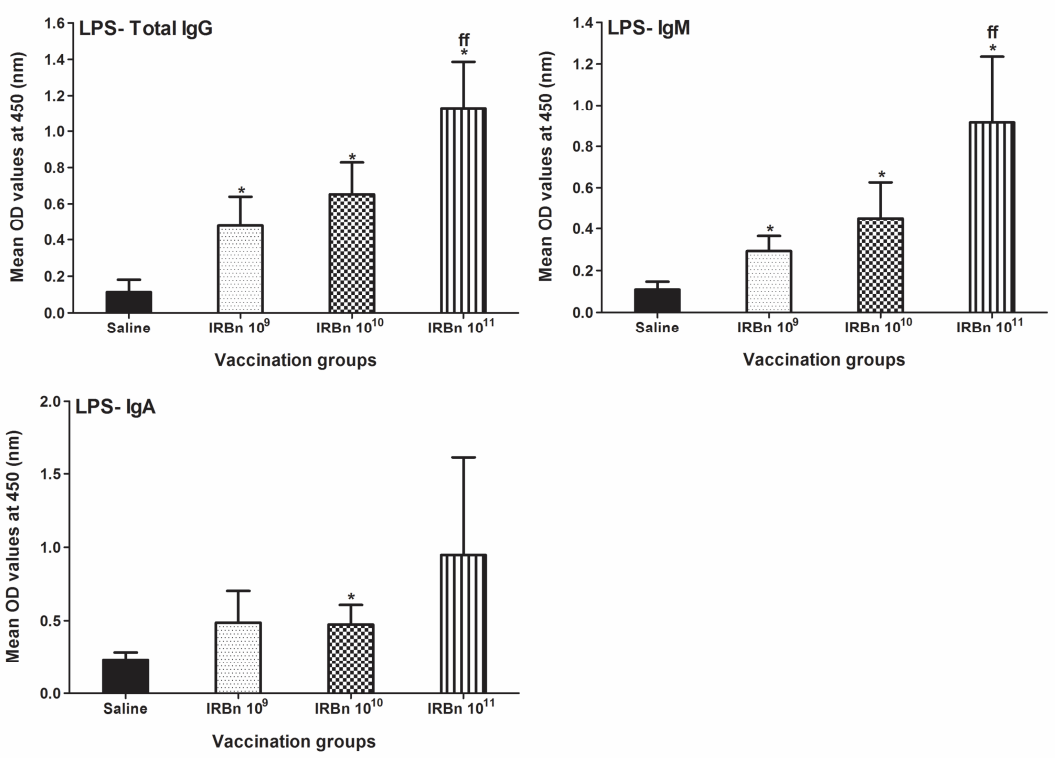

Figure 6.6. ELISA detection of IgG, IgM and IgA antibodies specific to *B. neotomae* LPS in the intestinal secretions of mice vaccinated with different doses of gamma-irradiated *B. neotomae*. Mice were vaccinated with 10^9 CFU-equivalent or 10^{10} CFU-equivalent or 10^{11} CFU-equivalent of gamma-irradiated *B. neotomae*, or inoculated with saline. Intestinal secretions were collected at 2 weeks after the last booster vaccination, were diluted 1 in 10 and assayed for the presence of LPS-specific antibodies. Results are shown as mean ± standard deviation (n = 4) of absorbance at 450 nm of the color developed. *Significantly different from the corresponding saline group ($P < 0.05$). ^{ff} Significantly different from the corresponding vaccination groups ($P < 0.05$). OD, optical density.

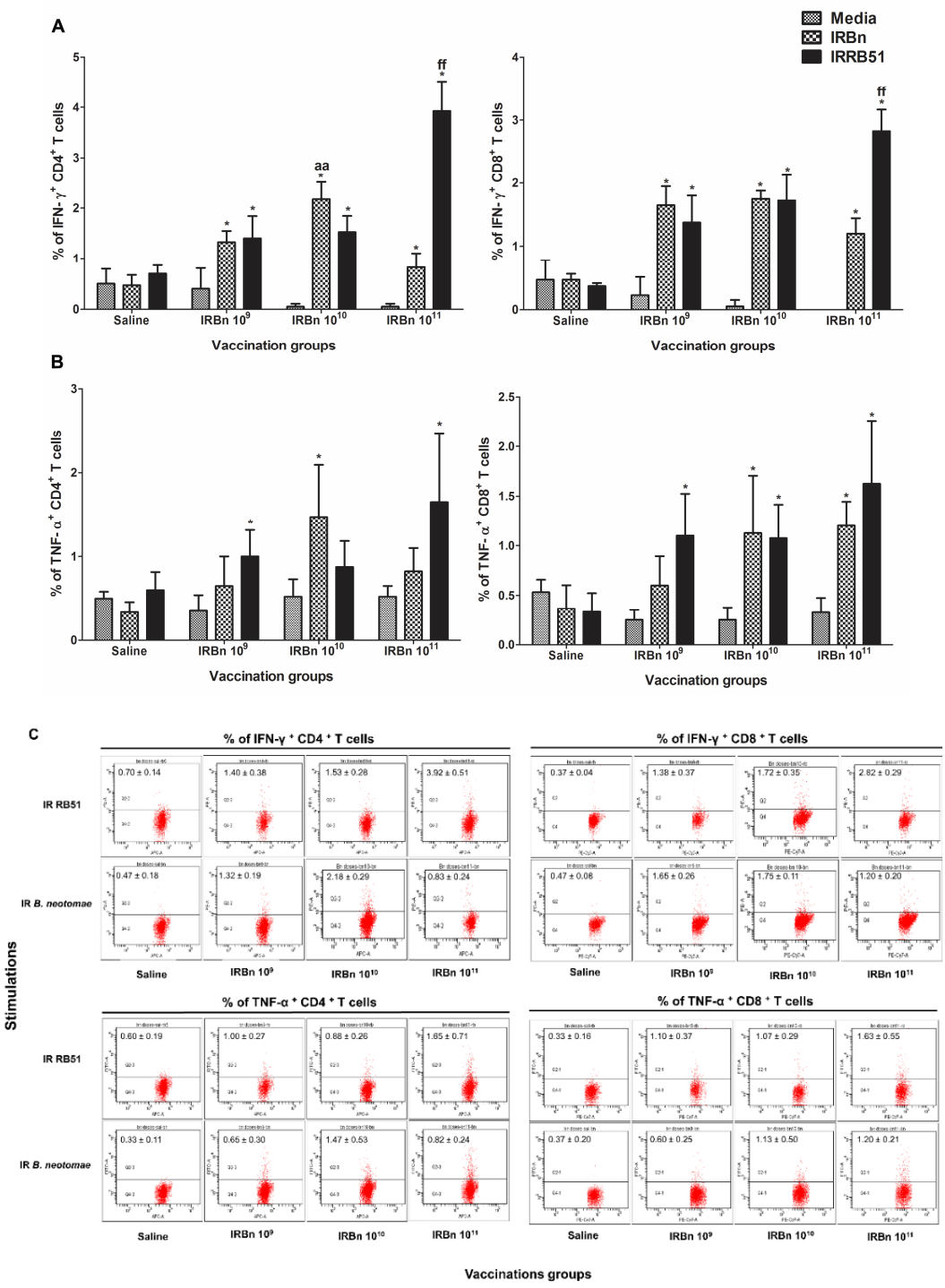

Figure 6.7. Flow cytometric analysis showing the percentage of (A) interferon-γ secreting, and (B) tumor necrosis factor-α secreting $CD4^+$ and $CD8^+$ T cells in the spleens of BALB/c

mice immunized with different doses of gamma-irradiated *B. neotomae*. Mice were immunized with 10^9 CFU-equivalent or 10^{10} CFU-equivalent or 10^{11} CFU-equivalent of gamma-irradiated *B. neotomae*, or inoculated with saline. Splenocytes from the vaccinated mice were stimulated with media (unstimulated), gamma-irradiated *B. neotomae* and gamma-irradiated RB51 and the percentage of IFN-γ secreting and TNF-α secreting $CD4^+$ and $CD8^+$ T cells were analyzed by flow-cytometry. (C) Representative figure of the flow cytometric analysis. *Significantly different from the corresponding unstimulated control within a vaccination group ($P < 0.05$). [aa] Significantly different from the corresponding vaccination groups with irradiated *B. neotomae* stimulation ($P < 0.05$). [ff] Significantly different from the corresponding vaccination groups with irradiated RB51 stimulation ($P < 0.05$).

CHAPTER 7. CONCLUSIONS AND FUTURE DIRECTIONS

Research conducted in this dissertation was aimed at developing improved vaccines for brucellosis using *B. abortus* RB51 and *B. neotomae*. Th1 type of cell-mediated immunity is crucial for vaccine-induced protection against brucellosis. In addition, antibodies to the O-polysaccharide (O-PS) of the lipopolysaccharide (LPS) play a protective role against infections with *B. abortus, B. melitensis* and *B. suis*. *B. abortus* RB51, an attenuated rough strain, is used as a live vaccine in the control of bovine brucellosis. Animals do not produce antibodies to the O-PS in response to the RB51 vaccination. We asked if engineering RB51 to synthesize O-PS and smooth LPS would enhance its vaccine efficacy. In this regard, we first tested whether overexpression of two glycosyltransferases WbkA and WbkE, which are essential for O-PS synthesis in *Brucella*, in RB51 results in O-PS synthesis and confers it a smooth phenotype. Quite unexpectedly, we found that overexpression of *wbkA* in RB51 leads to an extreme clumpy phenotype and synthesis of an exopolysaccharide (EPS). Our study, for the first time, demonstrates the novel role of *wkbA* in EPS synthesis. This finding extends the growing evidence for the genetic competence of *Brucella* to synthetize EPS. Lectin binding studies demonstrated that the exopolysaccharide contains mannose, galactose, N-acetylglucosamine and N-acetylgalactosamine. The EPS production resulted in enhanced adherence of the bacteria to polystyrene matrices. However, EPS did not change the attenuation characteristic of strain RB51WbkA. Also, our results indicate that EPS has minimal, if any, role in modulating immune responses in mice; the RB51WbkA vaccine induced a similar level of protective response against virulent *B. abortus* challenge as strain RB51. Future studies should focus on the translational implication of this finding; studies to determine how EPS affects bacterial fitness under different environmental conditions would help delineate the precise role of EPS during *Brucella* infection.

Purification and structural characterization of the EPS would also aid in the understanding of its possible function during infection. Lastly, as *wbkA* gene is a target of quorum sensing (QS)-regulators, it would be interesting to investigate the role of QS-regulators in altering the expression of *wbkA* under specific conditions and the effect on EPS synthesis. From practical point of view, it would be interesting to examine if EPS production has any benefit to RB51 vaccine manufacturing, such as increased stability during lyophilization or increased shelf-life of the vaccine.

The second objective was to determine whether increasing the amount of bactoprenol primed molecules in strain RB51WboA would increase the expression of O-PS and restore the smooth phenotype. Gene *wbkF* encodes undecaprenyl-glycosyltransferase enzyme that catalyzes the transfer of an acetyl-aminosugar to undecaprenylphosphate leading to the priming of this carrier for O-PS polymerization. Interestingly, *B. ovis* contains a non-functional *wbkF* while *B. canis* carries a deletion in the region encompassing *wbkF* and *wbkD*. In our study (Chapter 5), we overexpressed the *wbkF* gene in strain RB51WboA to generate strain RB51WboAKF. Our study indicates that strain RB51WboAKF produces smooth LPS but does not exhibit smooth colony phenotype or increased virulence. Immunization with strain RB51WboAKF induces enhanced antigen-specific antibody as well as cell-mediated responses, primarily of Th1 type, in mice. Also, strain RB51WboAKF confers a superior level of protection against virulent *B. abortus* 2308 and *B. melitensis* 16M than strain RB51 and RB51WboA in mice. These results are extremely promising and suggest that strain RB51WboAKF could be a more efficacious vaccine than its parent strain in natural hosts. Future studies should focus on determining the vaccine efficacy of RB51WboAKF in cattle and small ruminants against infections with virulent *Brucella* spp. An important consideration is that safety concerns may preclude the use of antibiotic resistant plasmids in vaccines. Alternate strategies to ensure plasmid maintenance need to be exploited for increasing the safety profile of the vaccine. One way to engineer an antibiotic resistance-marker free system is to construct an auxotrophic bacterial strain by mutating an essential gene. The growth of this strain will be restored by complementation or introduction of a plasmid carrying the deleted gene. Another important consideration that can affect the widespread use of this strain is the induction of anti-O-PS

antibodies. Most of the serodiagnostic assays for brucellosis are based on the detection of anti-O-PS antibodies. To prevent confounding serology, we will need alternate methods for differentiation of vaccinated animals from animals infected with wild-type *Brucella* spp. Previously, efforts have been directed towards modifying the O-PS of *Brucella* to express a distinct immunogenic epitope that does not react with the antibodies from naturally infected animals. Also, recent unpublished data proposes that O-PS of *Brucella* is capped by a M-specific tetrasaccharide sequence. Therefore, it is feasible to designing appropriate assays that utilize specific oligosaccharide antigens for the detection of antibodies in animals infected with wild-type *Brucella*. Our present study also suggests that, in addition to *wboA* gene, mutations affecting expression of several other genes involved in the synthesis of O-PS and smooth LPS contribute to rough phenotype of strain RB51. Identification of the other contributing mutations in RB51 will help to fully reveal the biosynthetic pathway of *Brucella* smooth LPS and its regulation in response to different microenvironments.

Human brucellosis is a major public health concern in several developing countries. Emergence of new foci of zoonotic *Brucella* spp. around the world has significantly changed the epidemiology of human brucellosis. There is a need to develop a safe and effective vaccine for use in humans. The third objective of the current study was to test the ability of orally inoculated gamma-irradiated *B. neotomae* and *B. abortus* RB51 in a prime-boost immunization approach to induce antigen-specific humoral and cell mediated immunity and protection against challenge with virulent *B. abortus* 2308. Our results indicated that heterologous prime-boost vaccination with *B. abortus* RB51 and *B. neotomae* and homologous prime-boost vaccination of mice with *B. neotomae* led to the production of serum and mucosal antibodies specific to the smooth LPS. Also, all oral vaccination regimens induced antigen-specific $CD4^+$ and $CD8^+$ T cells capable of secreting IFN-γ and TNF-α. Upon intra-peritoneal challenge, mice vaccinated with *B. neotomae* showed the highest level of resistance against virulent *B. abortus* 2308 colonization in spleen and liver. The next set of experiments with different doses of *B. neotomae* showed that all tested doses of 10^9, 10^{10} and 10^{11} CFU-equivalent conferred significant protection against the intra-peritoneal challenge. However, the highest tested dose of 10^{11} CFU-

equivalent of *B. neotomae* was required for affording protection against intranasal challenge as shown by the reduced bacterial colonization in spleens and lungs. Future studies should focus on a comprehensive analysis of T cell responses in lungs following immunization and challenge to reveal any important differences between the different booster-dose combinations. Additionally, determining the persistence of gamma-irradiated bacteria in lymphoid organs will be important to enhance our understanding of the magnitude of immunity induced at different mucosal sites following vaccination with different doses. An equally important future direction would be to study the effect of the number of boosters of the highest tested dose on the protective efficacy against mucosal and systemic challenge. Also, efforts should be focused on the formulation of vaccine to overcome the physiological barriers at mucosal routes including the acidic conditions of human stomach, and determination of the shelf-life of the gamma-irradiated vaccine and the duration of the vaccine-induced immune response.

Taken together, our studies discovered the novel role of *wbkA* in EPS synthesis in *Brucella*. Also, the recombinant strain RB51WboAKF is more effective than its parent strain in inducing protection in mice. The last part of the present research demonstrates the feasibility of using gamma-irradiated *B. neotomae* as an effective and safe oral vaccine to induce protection against respiratory and systemic infections with virulent *Brucella*.

VITA

VITA

EDUCATION

- BVSc & AH, College of Veterinary Science, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India, 2008

RESEARCH INTERESTS

- Infectious diseases; Host-pathogen interactions; Molecular bacteriology; Molecular diagnostics; Vaccines

JOURNAL PUBLICATIONS

- **N. Dabral**, M. M. Lafont, N. Sriranganathan and R. Vemulapalli. Oral immunization of mice with gamma-irradiated *Brucella neotomae* induces protection against intraperitoneal and intranasal challenge with virulent *B. abortus* 2308. *PLOS ONE* 9(9), e107180 (2014).
- **N. Dabral**, N. Jain-Gupta, M. N. Seleem, N. Sriranganathan and R. Vemulapalli. Overexpression of *Brucella* putative glycosyltransferase WbkA in *B. abortus* RB51 leads to production of exopolysaccharide (submitted).

ABSTRACTS

- M. M. Sanchez-Jimenez, **N. Dabral**, M. Olivera-Angel and R. Vemulapalli. Development of an ELISA test using recombinant proteins for the detection of *Brucella canis*. *4th Scientific Symposium of the International Infectious Diseases and Global Health Training Program, Infection*, 17(2), 111-120, Medellin, Colombia (2013).

SELECTED PRESENTATIONS

- **N. Dabral**, G. Kimsawatde, N. Sriranganathan, and R. Vemulapalli. Vaccination with recombinant *Brucella abortus* RB51 strain engineered to express increased levels of O-polysaccharide provides enhanced protection in murine brucellosis model. Brucellosis International Research Conference, Berlin, Germany, September, 2014
- **N. Dabral**, N. Sriranganathan, and R. Vemulapalli. Inducing O-polysaccharide expression in *Brucella abortus* vaccine strain RB51 to enhance its mmunogenicity. Phi Zeta Research Conference, West Lafayette, IN, April, 2014 **(honorable mention)**
- **N. Dabral**, N. Sriranganathan, and R. Vemulapalli. Better vaccines for brucellosis. Health and Disease: Science, Culture, and Policy Research Poster Session, West Lafayette, IN, March, 2014 **(poster award)**
- **N. Dabral**, N. Sriranganathan, and R. Vemulapalli. Overexpression of *wbkF* gene in *Brucella abortus* RB51WboA leads to increased O-polysaccharide expression. 66th Annual Brucellosis Research Conference - CRWAD, Chicago, IL, December, 2013
- **N. Dabral**, M. Moreno-Lafont, and R. Vemulapalli. Antigen-specic immune responses of mice to oral prime-boost immunization with gamma-irradiated *Brucella neotomae* and *B. abortus* RB51. 65th Annual Brucellosis Research Conference - CRWAD, Chicago, IL, December 2012
- **N. Dabral**, N. Jain, and R. Vemulapalli. Overexpression of a putative glycosyltransferase WbkA of *Brucella* in *B. abortus* RB51 leads to production of exopolysaccharide. Brucellosis International Research Conference, Buenos Aires, Argentina, December, 2011
- **N. Dabral**, V.K. Garg, D. Moustafa, N. Jain, C.B. Carlson, N. Sriranganathan, and R. Vemulapalli. Immunogenicity of a gene BMEI0123 encoding a putative outer membrane peptidyl-prolyl cis-trans isomerase of *Brucella*. 62nd Annual Brucellosis Research Conference - CRWAD,Chicago, IL, December, 2009

AWARDS

- **Honorable mention** for "Inducing O-polysaccharide expression in *Brucella abortus* vaccine strain RB51 to enhance its immunogenicity", presented at Phi Zeta Research Conference, April 2014, West Lafayette, IN
- **Poster award** for "Better vaccines for brucellosis", presented at Health and Disease: Science, Culture, and Policy Research Poster Session, March 2014, West Lafayette, IN
- Elected **member**, Phi Zeta Honor Society of Veterinary Medicine-OMICRON Chapter, Purdue University; inducted on April 11, 2011
- **Vice Chancellor's Gold Medal** in recognition for the highest CGPA in the class of 2008, June 2009, GBPUAT, India
- **University Certicate of Distinction** in recognition for the outstanding academic performance in DVM, June 2009, GBPUAT, India
- **Indian Herbs Animal Health Award** for academic excellence, August 2007, India
- **University Academic Merit Scholarship** for 5 consecutive academic years (2003-2008), GBPUAT, India