Brucella abortus Strain RB51 Outer Membrane Vesicles as a Vaccine Against Brucellosis in a Murine Model

by

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Abstract

Brucella abortus is a zoonotic agent that primarily infects cattle and causes brucellosis. *B. abortus* strain RB51 is a live, attenuated vaccine licensed for cattle. However, there is no available vaccine to prevent human brucellosis. Outer membrane vesicles have been tested as potential vaccines to prevent diseases caused by bacterial species. OMV are constantly released from Gram-negative bacteria. They are comprised principally of the outer membrane components and periplasmic proteins from the bacterial cell envelope. The research in this thesis examined the adjuvant property of non-replicative, metabolically active irradiated strain RB51 and the protective ability of OMV derived from strain RB51. Irradiated *B. abortus* strain RB51 was assessed for its ability to act as an adjuvant to induce protection against malaria. It was found that irradiated *B. abortus* strain RB51 administered along with fasciclin related adhesive protein (FRAP) to mice induced a protective immune response and a significant decrease in parasitemia after challenge with *Plasmodium berghei*. Strain RB51 and strain RB51 over-producing Cu/Zn superoxide dismutase (Cu/Zn SOD) were used to produce OMV. Western blotting and SDS-PAGE gel staining confirmed the presence of OMV and the over-production of Cu/Zn SOD. OMV were delivered to mice using an intraperitoneal route and, in some cases, with aluminum hydroxide adjuvant. The immune response was assessed by antibody isotyping with respect to OMV and measuring splenic clearance (i.e. protection) from a *B. abortus* strain 2308 challenge. The results demonstrate that

OMV from *B. abortus* strain RB51 or strain RB51 over producing Cu/Zn SOD produced a Th1 polarized immune response as measured by specific OMV antibodies and cytokines but no statistically significant protection was observed.

Dedication

I would like to dedicate this thesis to my parents, Mr. Clifton Cassidy and Mrs. Cindy Cassidy. Nothing I have done in my life would have been possible without them. They have always pushed me to be my best and to succeed in life. This work is a product of the faith and hope that they have always had in me.

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Last but certainly not least, I want to thank my parents. My parents are, and always have been, the driving force in my life. They have been with me every step of the way. I could not ask or hope for better role models than I have been given. My life is continually improved and enriched by my parents. Words cannot express how much I owe to my parents. I love them with all of my heart and I truly am the luckiest person in the world to have them in my life. Thank you Mom and Dad!

Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 1: Literature Review

OMV Background

Outer membrane vesicles (OMV) were identified almost 40 years ago in *Pseudomonas aeruginosa* exposed to N, N'-dimethylformamide (DMF) (93, 94). The vesicles released from *P. aeruginosa* were described as blebs and bleb-like, a nomenclature that is still used to this day, however, OMV is much more widely used. OMV are products of Gram-negative bacteria and are comprised principally of the outer cell membrane, lipopolysaccharide (LPS), and periplasmic proteins (12, 57). OMV have been shown to be a stress coping mechanism and a way to rid the cell of older or misfolded outer membrane proteins (OMP) and periplasmic proteins (58, 59). OMV are constitutively released from Gram-negative bacteria during log phase (12, 13, 70). The overall dimension of OMV, 50 to 250 nm, is comparable in size to the synthetic nanoparticles (1). A wide range of functions has been proposed for the OMV including export of virulence factors and transfer of antibiotic resistance genes (12, 21, 40, 43). OMV are very complex particles e.g. in terms of the number of proteins and carbohydrates, and can obviously have very pleiotropic effects inside a host.

During the release of OMV from Gram-negative bacteria, the outer membrane looks similar to that of an apoptotic mammalian cell. The outer membrane begins to bulge outward and protrude away from the inner membrane. A membrane fission process then occurs, incorporating periplasmic components into the budding OMV, and encloses the bulging outer membrane to generate the complete OMV. Production of OMV has

been observed in bacteria growing in the planktonic or biofilm mode, on solid or liquid media, in swarming cultures and in natural environments, including infected tissues (12, 49, 59, 93). In a pioneering study on OMV, only the cleaved, mature form of proelastase was found in OMV from *P. aeruginosa* while the cleaved form is found normally in the periplasm of the bacterial species (12). The thought that only periplasmic proteins are incorporated into OMV was confirmed because the larger precursor of the proelastase, found only in the cytoplasm, was not present in the OMV.

OMV can be easily isolated from Gram-negative bacteria growing in liquid or on solid media (85, 108). Isolation of OMV can be accomplished through differential centrifugations that separate the OMV from the planktonic bacteria. Protocols using centrifugation have been shown to be effective in isolating OMV in most Gram-negative bacteria including, but not limited to *Neisseria* spp., *Vibrio* spp., and *Pseudomonas* spp. (40, 67, 85, 91, 93, 94).

LPS is constitutively produced in Gram-negative bacteria and present in OMV of both smooth and rough type Gram-negative bacteria. Concentrations of LPS found in OMV are not affected by the phenotype of the bacteria, i.e. rough or smooth (12, 43, 94). LPS is an activator of innate immunity through the Toll-like receptor 4 (TLR4) pathway and in large amounts can cause a toxic effect in hosts infected with Gram-negative bacteria (17). The toxicity of LPS is especially noted in the family *Enterobactericae*. Hence, antigenic differences in LPS mutants that make the molecule less toxic have been looked at in some species as potential vaccine candidates (e.g. *N. meningitidis*) (6, 9).

Outer membrane proteins (OMP), or lipoproteins, are also present in OMV. OMP have been shown to activate innate immunity through a TLR2 dependent pathway (71) and could translate into an enhanced means for OMV to serve as vaccines.

OMV Vaccines

OMV vaccines have been in development for more than 20 years (107). The most common vaccine preparation for OMV is used in immunization against *N. meningitidis* infections in humans (8, 36, 79). OMV vaccines have also been developed towards *Vibrio cholerae*, *Bordetella pertussis*, and other Gram-negative pathogens (77, 85, 89). OMV based vaccines consist of OMV isolated using traditional methods (10, 85). The OMV have been delivered in many different ways including intranasal, intraperitoneal, intragastric and all induce measurable levels of protection (32, 77, 85).

In the case of *N. meningitidis*, there are different serogroups within the species that have not been shown to produce cross protective antibodies after vaccination, using various methods and routes of inoculation (26, 54). Using OMV from multiple serotypes or a multivalent OMV could circumvent this problem (54, 80). Theoretically, if one were able to engineer multivalent OMV for different serotypes of the same organism, it should be possible to engineer a multivalent vaccine to target two different species of Gram-negative bacteria. Moreover, the possibility exists of using genetically engineered Gram-negative bacteria to produce protective antigens in OMV to target organisms that are completely unrelated (e.g. Gram-positive bacteria, viruses, and parasites).

OMV elicit a wide variety of cytokines and antibodies in the immunized host. *N. meningitidis* OMV stimulate proinflammatory cytokines and chemokines including tumor necrosis factor- alpha (TNF-alpha), interleukin-1beta (IL-1beta), interleukin-6 (IL-6), and interleukin-8 (IL-8). Cytokine stimulation from exposure to OMV has been proposed to be a preliminary step in setting up meningococcal pathogenesis. However, innate immunity activation could lead to protective immunity when vaccinating with meningococcal OMV (46, 61). TNF-alpha production by granulocytes and monocytes is dependent on TLR2 and TLR4 pathways during exposure to meningococcal OMV (62). OMV vaccines have also been shown to elicit strong, neutralizing serum and mucosal (i.e. IgM, IgG, and IgA) antibody responses (38, 110).

Malaria Prevalence and Symptoms

Malaria is considered the most deadly parasitic disease in the modern world (39). *Plasmodium falciparum* is the etiologic agent of malaria (25). *P. falciparum* infects 300 to 500 million people a year, causing approximately 1 million deaths each year. The parasite has developed resistances to some of the most common drugs used to treat it, and no vaccine exists for *P. falciparum* to aid in prevention (35).

Clinical diagnosis for malaria can be effectively achieved through microscopy. However, many clinical settings where malaria is prevalent do not have access to microscopy facilities. The lack of accurate means for diagnosis typically leads to misdiagnosis (35). Uncomplicated malaria presents clinically in children as fever, vomiting, and diarrhea, conditions that are typically of most other childhood ailments.

However, malaria in adults can cause multiple organ failure, including renal failure. The difference between clinical presentation in young and adult individuals is not fully understood in malaria pathogenesis (35).

Brucella Etiology and Pathogenesis

Bovine brucellosis is caused by a facultative, intracellular bacterial pathogen known as *Brucella abortus* (44). Transmission can occur vertically, through contaminated milk, or through contaminated abortion secretions, the latter of which being the most important in cattle (18). Infection can result from contact with skin, conjunctiva, or respiratory mucosa, but the most common means of infection occurs through the bovine respiratory tract (18). Brucellosis presents clinically in cattle by causing abortions or producing weak, newborn calves (18). However, symptoms of infection vary greatly between host species when infected with the intracellular pathogen (53). Differences between clinical manifestations of different species are not fully understood (53). The outcome of infection in cattle is highly dependent on age, reproductive and immunological status, natural resistance, and strain infectivity (2). Infection is often inapparent in bulls, but can lead to fever, anorexia, and depression (18). The most common lesion in *Brucella* infected bulls is orchitis, which can result in permanent infertility (60, 74).

Abortion typically occurs in cattle during the last trimester of gestation (18) when *B. abortus* migrates to the uterus. High levels of erythritol and steroid hormones secreted by trophoblasts surrounding the gravid uterus are likely the cause of this migration (18).

Erythritol is conducive to *Brucella* survival because *B. abortus* can use it as both a carbon source and an energy supply (81). Multiplication of the *Brucella* causes an influx of inflammatory cells into the fetal placental area (81). Metabolic exchanges are compromised due to this influx of cells resulting in fetal abortion (81). After the initial abortion, most cows are able to progress to full parturition upon subsequent pregnancies, although another abortion is possible (18).

Brucella Immunity

B. abortus activates innate and acquired immunity *in vivo* over the course of infection (44). Innate immunity is the first line of defense against *Brucella* spp. infection. *B. abortus* LPS mediates the interaction between *B. abortus* and complement component in the host. Complement components are found in the serum and are responsible for early serum bactericidal activity. Smooth *B. abortus* are more resistant to serum bactericidal activities. *B. abortus* LPS obtained from smooth strains does not activate the alternative complement pathway. In fact, the classical, IgM-mediated complement pathway is the most prevalent way in which *Brucella* spp. activate complement in the host. Neutrophils rapidly phagocytize *B. abortus* after opsonization in the serum. Neutrophils likely serve as a transport vesicle for *B. abortus* to lymphoid tissues. Macrophages are activated by IFN-γ and TNF-α. The macrophages then phagocytize *Brucella* and exhibit bactericidal activity due mainly to myeloperoxidase-H_2O_2-halide activity during oxidative burst. However, some of the *Brucella* survive and the host must rely on the cellular immune system to clear the pathogen (44).

Cellular immunity is responsible for *Brucella*-specific immune responses and critical for vaccine-mediated protection (44). $CD8^+$ T cells are critical in controlling *Brucella* infection. $CD8^+$ T cells are responsible for killing *Brucella*-infected macrophages. $CD4^+$ T cells, $CD8^+$ T cells, and macrophages secrete IFN-γ and IL-2 to help drive immune system polarization to a Th1 mediated response. B cell production switches to IgG2a and IgG3 isotypes to facilitate opsonization. Opsonization induces phagocytosis of extracellular bacteria and helps control *Brucella* infection (44).

Both innate and adaptive immunity are important in controlling *Brucella* infection (44). Th1 polarization of the adaptive immune system is crucial in the host controlling infection. Vaccination can enhance the protection afforded by the host's immune system due to priming of the adaptive immune system (44).

Brucella as an Adjuvant

An adjuvant is an immunological agent that is used to augment the host's immune response towards a specific antigen. Adjuvants can consist of many different types of entities, from individual peptides to oil emulsion mixtures. *Brucella* peptides and killed *Brucella* are documented to have adjuvant capabilities (30, 78, 88). Specifically, Golding et al. has shown that heat killed *B. abortus* conjugated to human immunodeficiency virus (HIV) peptides have been able to induce antibody mediated immunity and antiviral activity. The HIV antigen was conjugated to the membrane of the heat killed *B. abortus*. The vaccine stimulated mucosal antibody mediated immunity to produce antiviral effects

(30, 88). *B. abortus*'s adjuvant capabilities make it a very attractive option for vaccines that need a strong immune response.

Brucella **Vaccines**

B. abortus vaccines have traditionally been based on live, attenuated *B. abortus* (87). *B. abortus* strain 19 is attenuated and unable to grow in the presence of erythritol (11, 87). However, the presence of the O-side chain caused vaccinated animals to become seropositive thus confounding serodiagnosis since vaccinated animals could not be differentiated from field-infected animals. The production of antibody against the O-side chain due to vaccination with strain 19 makes this strategy unfavorable for use as a vaccine considering the current test and slaughter practices used to control brucellosis in cattle (87).

B. abortus strain 45/20 was utilized in the first attempt to use a rough, live, attenuated vaccine to protect against bovine brucellosis (87). Attenuation was achieved through serial passage of the *B. abortus* 45/0 field isolate in a guinea pig 20 times. The resulting strain 45/20 was able to protect guinea pig and cattle from brucellosis. However, the strain would revert to a smooth phenotype *in vivo*, and offset the purpose of developing the rough strain (87) i.e. avoidance of confounding serodiagnosis.

B. abortus strain RB51 was developed at Virginia Polytechnic Institute and State University during the 1980's and conditionally approved by the United States Department of Agriculture (USDA) for use in cattle in 1996 (86, 87) and unconditionally

approved in 2001. Multiple passages of *B. abortus* strain 2308 on rifampicin containing medium created the rifampicin resistant, rough strain. Strain RB51 produces little to no O-side chain in its LPS. The vaccine is a live, attenuated vaccine and has never been licensed for human use (87). Other rough *Brucella* vaccine strains have been created, *B. melitensis* VTRM1 and *B. suis* VTRS1, by deleting a large segment of the *wbo*A gene encoding a glycosyltransferase involved in O-side chain biosynthesis in the parent of each strain. Strains VTRM1 and VTRS1 did protect against challenge but did not have the same degree of attenuation as strain RB51 (87, 102). This indicates that *wbo*A encodes a potential virulence factor, but other genes are also affected in strain RB51, in addition to the IS711 insertional inactivation of *wbo*A, that allow a higher level of attenuation (87).

Perspective

OMV are natural products of Gram-negative bacteria that are comprised of different outer membrane components, lipoproteins, periplasmic enzymes and carbohydrates. OMV are approximately 50-250 nm in size and these vesicles are thought to be responsible for shedding of old or nonfunctional outer membrane components in stressed and non-stressed conditions, among other possible functions.

OMV have also shown to be useful to microbiologists and clinicians in terms of developing vaccines. Not only have OMV vaccines been developed, they have been shown to be effective, safe, and protective against serious human diseases (15, 22, 67). OMV should be able to deliver over-expressed, periplasmic and recombinant antigens. Moreover as a biodegradable delivery system capable of activating innate immunity,

OMV could stimulate acquired immunity against the recombinant antigen of choice. When one is dealing with Gram-negative pathogens that are viewed as too dangerous or risky to deliver as live, attenuated vaccines, OMV based vaccines could be of particular interest.

An effective *B. abortus* based OMV vaccine could be used as a human vaccine or as a safer bovine vaccine than the vaccines currently used to protect against bovine brucellosis. The studies in this thesis focus on using *B. abortus* strain RB51 as a killed adjuvant to improve protection against malaria or strain RB51 derived OMV to produce an effective vaccine against wild-type *B. abortus* strain 2308 infection in mice.

Chapter 2: *Brucella abortus* as an Adjuvant for the FRAP Protein of *Plasmodium falciparum*

Introduction

Malaria has become a worldwide problem and is the most lethal parasitic disease (39, 51). *P. falciparum* infects 300-500 million people annually (25), and despite extensive efforts, no vaccine has been established that is effective at controlling the spread of malaria (75). Therefore, a vaccine capable of generating a strong, protective immune response targeting an essential malarial peptide is needed to help control the spread of the pathogen.

Fasciclin Related Adhesive Protein (FRAP) is responsible for hemoglobin degradation upon replication of the malaria parasite inside a red blood cell (RBC) (39, 75). The hemoglobin molecule makes up 90% of the total protein found in RBC. Without the FRAP protein, the intracellular RBC environment would be toxic towards *P. falciparum* and the parasite would not be able to survive (39, 106). Colonization of red blood cells by *P. falciparum* causes the onset of clinical symptoms associated with malaria (39). A vaccine capable of eliciting a strong immune response towards the FRAP antigen may be able to control disease and prevent clinical signs.

In the present study, a FRAP based subunit vaccine is used in the mouse model to determine the vaccine's protective ability. The vaccine is mixed with irradiated *B.*

abortus strain RB51 as a free adjuvant in order to stimulate a protective immune response against challenge with the mouse parasite *P. berghei*. Heat-killed *B. abortus* has previously been shown to be an effective adjuvant when conjugated to certain Human Immunodeficiency Virus (HIV) peptides (30, 45, 88). *B. abortus* strain RB51 has also been shown to be an effective carrier for heterologous antigens able to induce protective immunity (73, 101, 105). The data presented in these studies suggests that irradiated *B. abortus* strain RB51 could serve as a suitable adjuvant for the FRAP subunit vaccine.

Materials and Methods

Bacterial Strains: *B. abortus* RB51

Irradiation

B. abortus strain RB51 was grown for 23 hours at 37°C in trypticase soy broth (TSB) to a density of 10^8 cfu per ml. The cells were centrifuged at 1,000 x g for 10 minutes and then washed with phosphate buffered saline (PBS). The cells were resuspended in PBS at a concentration of 10^9 cfu per ml. One ml of the resuspended cells was exposed to 440 kilorads using a model 109 cobalt irradiator (J.L. Shepherd and Associates, San Fernando, CA). After irradiation, the culture was plated on trypticase soy agar (TSA) and incubated for three days at 37°C to determine whether strain RB51 had been rendered replication deficient.

Vaccines: subunit and strain construction

The vaccines used in this study are listed in Table 1 (pg. 49). The vaccines were delivered as 100 μl aliquots to each mouse intraperitoneally (i.p.). All mice received 20 μg of FRAP protein (Dharmendar Rathore, Virginia Bioinformatics Institute, Blacksburg, VA) for each vaccination and boost. Irradiated strain RB51 was delivered to the mice at 10^8 cfu for the first vaccination and 10^4 cfu for each boost. Complete Freund's adjuvant (Invitrogen, Carlsbad, CA), 50 μl of 1 mg/ml heat killed *Mycobacterium tuberculosis*, was used for the first vaccination and incomplete Freund's adjuvant (Invitrogen, Carlsbad, CA), 50 μl, was used for each boost.

Vaccine immunizations

CD-1 female mice were received from Charles River Laboratory (Wilmington, MA) at 4 weeks of age. All mice were acclimated for two weeks in the animal housing facility of the Infectious Disease Unit in the College of Veterinary Medicine, as approved by IACUC #08-070-CVM. Vaccines were delivered i.p. and the first boost at three weeks post-vaccination. The second boost was given at 3 weeks post-boosting. Mice were bled retro-orbitally two weeks post each vaccination to harvest blood. Whole blood was centrifuged at 1,931 x g to isolate serum and allow for assessment of antibody levels specific for FRAP protein.

ELISA assay for antibody titrations

Enzyme-linked immunosorbance assays (ELISAs) were performed with standard methodologies (92) using 50 μl of FRAP protein at a concentration of 2.5 μg/ml for the coating antigen. Fifty μl of mouse serum at a dilution of 1:100 from the vaccinated mice was used to bind to the coating antigen and 50 μl of secondary anti-mouse serum conjugated to HRP (ICN, Solon, OH) at a dilution of 1:5000 was used for color development. TMB membrane peroxidase substrate (KPL, Gaithersburg, MD) was added to the plate to cause color production and TMB stop solution (KPL, Gaithersburg, MD) was added to stop the reaction. The ELISA plates were read at 450nm and an OD value was obtained in a microplate reader (Versamax, Molecular Devices, Sunnyvale, CA).

Challenge of mice with *Plasmodium berghei* and assessment of protection.

All mice were challenged i.p. with *P. berghei* prepared in whole blood (Dharmendar Rathore, National Institutes of Health, Bethesda, MD) at a concentration of 10^6 parasites at two weeks after the final boost. Parasitemia was assessed at 3, 5, 7, and 10 days post challenge by examining blood smears prepared with Giemsa stain; 1000 RBC were examined at a magnification of 400x and a percent parasitemia calculated by dividing parasitized red blood cells by 1000.

Statistical Analysis

All data were analyzed by two-way analysis of variance using Prism software (GraphPad Software, La Jolla, CA). A P value of <0.05 was considered significant.

Results

Serologic Response

Rationale: ELISA was performed on all serum samples to assess whether the vaccinated mice were developing a specific immunoglobulin response towards FRAP protein.

As detected by ELISA, sera obtained from mice at 2 weeks post-vaccination and 2 weeks post-boosting contained no IgG1 or IgG2a antibody specific for FRAP (Fig. 1). However, sera from 2 weeks after the second boost of Freund's adjuvant and FRAP showed a significant increase in the amount of IgG1 and IgG2a compared to sera from saline vaccinated controls. A higher amount of IgG1 was present in the sera of these mice when compared to IgG2a levels. Sera from mice vaccinated with irradiated strain RB51 and FRAP also showed a significant increase in IgG1 and IgG2a at this time compared to saline vaccinated control mice. A higher amount of IgG2a was present in the sera of mice vaccinated with irradiated strain RB51 and FRAP compared to IgG1 levels. Sera from mice vaccinated with saline and FRAP showed a significant increase in IgG2a levels at this time compared to saline vaccinated controls, but no significant increase in IgG1 levels compared to saline vaccinated controls.

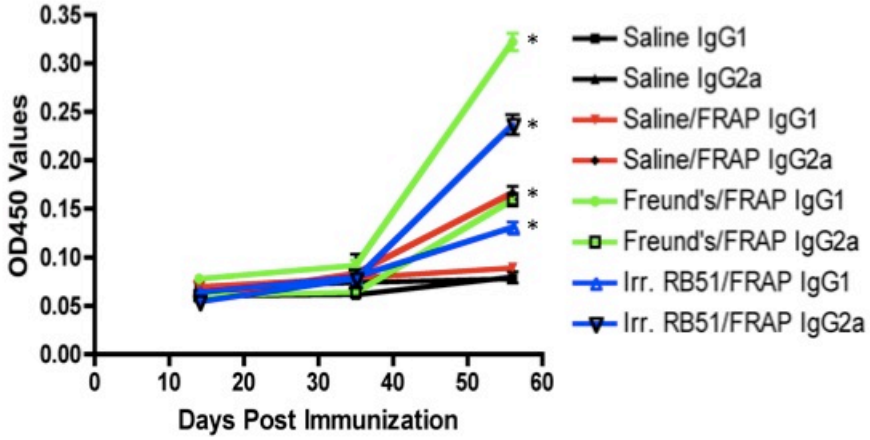


Figure 1. ELISA results read at OD_{450} for development of IgG1 and IgG2a antibody subtype towards FRAP. Significance was determined in all cases by two-way analysis of variance and a *P* value of ≤ 0.05 was considered significant compared to the negative control as measured at each time point and indicated by a *.

Measurement of *P. berghei* parasitemia in whole blood

Rationale: Protection experiments were performed to determine if irradiated strain RB51 used as an adjuvant in conjunction with FRAP provided a protective immune response against *P. berghei* challenge.

The immunized groups of mice were challenged at 2 weeks post-booster vaccination with *P. berghei* i.p. (Fig. 2). Groups vaccinated with Freund's adjuvant and FRAP protein and irradiated strain RB51 and FRAP protein showed a significant decrease in parasitemia when compared to saline control and saline with FRAP protein vaccinated groups. However, the group vaccinated with saline and FRAP protein also showed a significant decrease in parasitemia compared to saline controls at 7 and 10 days post challenge.

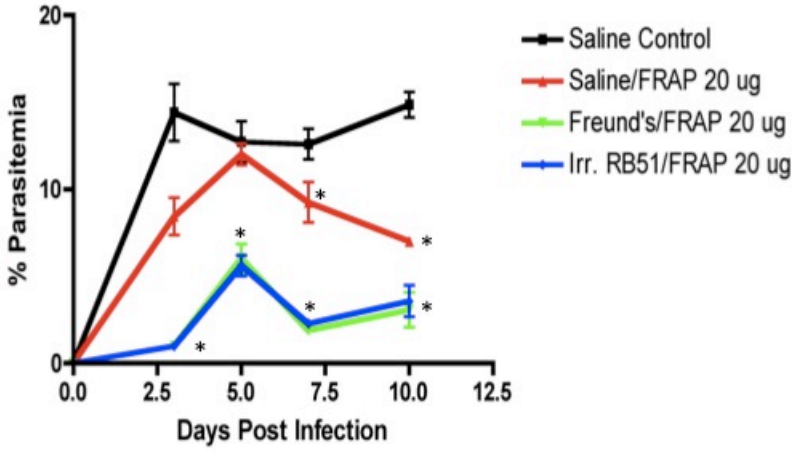


Figure 2. Parasitized RBC after infection measured at 0, 3, 5, 7, and 10 days post challenge. 1000 RBCs were examined at 400x magnification and percent parasitized RBCs determined. Significance was determined in all cases by two-way analysis of

variance and a P value of ≤ 0.05 was considered significant compared to the negative control as measured at each time point and indicated by a *.

Discussion

Previous studies have demonstrated that replication deficient (heat-killed) *B. abortus* can serve as an effective adjuvant producing protective immunity in the mouse model (30, 31, 45). In the current study, based on measuring the IgG isotypes stimulated, irradiated *B. abortus* strain RB51 was shown to be effective at eliciting an antibody mediated immunity towards a malarial protein FRAP. Furthermore, use of FRAP and irradiated *B. abortus* strain RB51 as an adjuvant was able to provide protective immunity against malarial infection in the outbred CD-1 mouse.

FRAP is an attractive antigen for a malarial vaccine because it is essential in the process of the parasite surviving within the RBC (39, 106). FRAP is responsible for detoxifying hemoglobin in the intracellular environment of the RBC (39, 75, 106). Since clinical signs of malaria are attributed to this stage of infection (39), stopping the detoxification of hemoglobin performed by FRAP could be key in controlling the disease (39, 75, 76).

The IgG subclass profiles in response to FRAP with irradiated strain RB51 as an adjuvant exhibited much higher levels of IgG2a than IgG1. Higher levels of IgG2a could indicate that irradiated strain RB51 is driving the immune response against FRAP to a Th1 polarization. However, a Th1 polarization cannot be determined without studies examining cytokine profiles. High levels of IgG2a are indicative of the adjuvant characteristics of *B. abortus* while presenting an antigen, in this case FRAP (30, 88). In

contrast, FRAP with Freund's adjuvant was able to induce higher levels of IgG1 than IgG2a against the antigen. As an adjuvant irradiated strain RB51 was able to induce a Th1 polarized response against FRAP, and Freund's adjuvant (complete and incomplete) was able to induce a Th2 polarized response against FRAP (90). Both adjuvants were able to elicit a significant immune response towards FRAP in a murine model, but the type of antibody subclass response differed between the two groups of mice.

Mouse models of malaria are very important tools for studying malarial infection. However, *P. falciparum* cannot cause disease in the mouse model. Genetically similar rodent malarial parasites, such as *P. berghei,* are therefore important tools in studying malarial pathogenesis in the mouse model (50).

Blood stage parasitemia is an important measure of a vaccine's efficacy against malarial infection (96, 109). In this study, irradiated *B. abortus* strain RB51 was used as an adjuvant in a malarial vaccine. Irradiated strain RB51 with FRAP induced a strong Th1 type response (90) and provided protective immunity against malarial infection similar to that of previous mouse model malaria studies (96, 109). Freund's adjuvant used with FRAP also provided a similar protective immune response compared to irradiated strain RB51. But protection in this case was due to a Th2 type response (90, 96, 109). However, Freund's adjuvant cannot be used in human vaccines (90). More studies showing the safety of irradiated strain RB51 could be performed to further validate approving irradiated strain RB51 as a safe and effective adjuvant for human vaccines.

The study presented in this thesis shows for the first time that irradiated *B. abortus* strain RB51 can serve as a safe and effective adjuvant to vaccines targeting pathogens. Irradiated *B. abortus* strain RB51 could be an ideal adjuvant for malarial vaccines. Not only would it provide a protective immune response towards malarial infection, but it could also provide protective immunity against *Brucella* infection (65, 82). Further studies using irradiated *B. abortus* strain RB51 could help elucidate its true potential as an adjuvant.

Chapter 3: Outer Membrane Vesicle Based Vaccines

Introduction

B. abortus is the etiologic agent of brucellosis, a disease that primarily affects cattle (33). Brucellosis is also the most common zoonoses in the world, and is typically transmitted to humans through contact with infected animals (7, 27, 33). Although brucellosis has become a major problem in the modern world, no approved human vaccines exist (4, 52).

Brucella spp. establish infection in humans by invading macrophages and avoiding host immune responses in order to establish chronic infection (27). The incubation period for disease in humans can vary from 5 days to 5 months. Disease in humans is typically known as Maltese fever or undulant fever (27, 29, 52). The most common clinical symptoms in human disease are recurring fever, headache, fatigue, malaise, weight loss, and night sweats (27, 52). Less common symptoms include abortions in pregnant females, endocarditis, and neurobrucellosis (27).

Outer membrane vesicles (OMV) have been shown to be safe and effective vehicles for human vaccines in prevention of diseases ranging from those caused by *Vibrio cholerae* to *Neisseria meningitidis* (5, 48, 77, 84). OMV are shed constitutively by most Gram-negative bacteria and are comprised of the outer membrane, LPS, and

periplasmic constituents of the bacteria (56, 84). OMV vaccines have proven to be safe for human, and they have also proven to be highly immunogenic (20, 34, 84, 85).

In the present study, due to previous success of OMV based vaccines (77, 85) and knowledge that *Brucella* spp. produce OMV (A. Contreras, Mexico, personal communication) (14, 28, 98), OMV from *B. abortus* strain RB51 were analyzed as a possible vaccine candidate against brucellosis in the mouse model. Immunogenicity and protective ability of the vaccine was examined in the mouse model in hopes of developing a cattle vaccine completely incapable of causing brucellosis and possibly a safe human vaccine against brucellosis.

Methods and Materials

Bacterial Strains: *B. abortus* 2308, *B. abortus* RB51, *B. abortus* RB51/SOD

Isolation of OMV

Cultures of *B. abortus* strain RB51 and strain RB51/SOD were grown overnight at 37^{o}C to approximately 350 Klett units measured with a Klett-Summerson photoelectric colorimeter in 1 liter of *Brucella* broth (Difco, Detroit, MI) supplemented with yeast extract or *Brucella* broth supplemented with yeast extract and 25 μg/ml chloramphenicol, respectively. The cultures were centrifuged at 1,931 x g for 10 minutes and the supernatant was filtered through a 0.22-micron filter. The filtered supernatant was checked for viability by streaking on trypticase soy agar (TSA) plates and then centrifuged at 176,509x g for 4 hours at 4^{o}C in an Optima L-90K Ultracentrifuge (Beckman Coulter, Brea, CA). The pellet was resuspended in 1 ml of sterile saline and stored at -20^{o}C until needed. Protein concentration was determined using a Bio-Rad DC Protein Assay Kit I (Bio-Rad, Hercules, CA) and the resulting color was read at OD_{750} in a Versamax microplate reader (Molecular Devices, Sunnyvale, CA).

Electron Microscopy

100 μl of strain RB51 OMV or strain RB51/SOD OMV were suspended in 900 μl of 37% formalin fixative (Fisher Scientific, Worcestor, MA). The concentration of OMV was either 80 μg/ml or 8 μg/ml. The samples were suspended in 2% aqueous uranyl acetate solution for negative staining and placed onto 200-mesh formvar carbon coated,

copper grids (Electron Microscopy Sciences, Hatfield, PA) (20). Excess liquid was wicked away and samples were viewed at 63,000x and 100,000x magnification on a Zeiss 10CA Transmission Electron Microscope (Virginia-Maryland Regional College of Veterinary Medicine).

Immunoblot assay

150 μg of OMV obtained from *B. abortus* strain RB51 and *B. abortus* strain RB51/SOD were treated with 40 μl of 2X Laemmli Buffer (Bio-Rad, Hercules, CA) and run on 10% SDS-PAGE gels (Invitrogen, Carlsbad, CA). Electrophoresis was performed at room temperature with a constant voltage of 165V for 50 minutes. After electrophoresis, the proteins from one gel were stained overnight with Coomassie blue to visualize the proteins. The proteins from the second gel were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) by applying a current of 15V for 45 minutes on a semi-dry transfer cell (Bio-Rad, Hercules, CA); the membrane was incubated with a polyclonal goat antibody directed against *Brucella* spp. Cu/Zn SOD (99). Immunochemical detection was performed using horseradish peroxidase (HRP)-conjugated mouse anti-goat immunoglobulins (ICN, Solon, OH) as the secondary antibody.

Vaccine strain construction

The vaccines used in this study are listed in Table 2 (pg. 49). The vaccines were delivered as 100 μl aliquots to each mouse intraperitoneally (i.p.) Each mouse received

15 μg of OMV protein for either strain RB51 or strain RB51/SOD vaccination. Aluminum hydroxide adjuvant (Sigma-Aldrich Corp. St. Louis, MO), 650 μg, was used for both the primary vaccination and the booster in its respective groups.

Vaccine immunization

BALB/c female mice were received from Harlan Laboratories (Dublin, VA) at 4 weeks of age. All mice were acclimated for two weeks in the animal housing facility at the Infectious Disease Unit in the College of Veterinary Medicine, as approved by IACUC # 08-070-CVM. Vaccines were delivered i.p. and boosts were given to each mouse at two weeks post-initial vaccination. Mice were bled retro-orbitally two weeks post each vaccination to check for antibody levels specific for strain RB51 OMV.

Challenge of mice with *Brucella abortus* 2308 and collection of tissues

Two weeks post-boost immunization all mice were challenged i.p. with 6.6×10^4 cfu of *B. abortus* strain 2308. Mice were euthanized by CO_2 asphyxiation and spleens were obtained from all mice at two weeks post-challenge. Spleens were homogenized using tissue grinders, serial diluted in sterile saline, and plated on trypticase soy agar (TSA) plates; splenic cfu were determined after incubation at 37°C for 5 days.

ELISA assay for antibody titrations

Enzyme-linked immunosorbance assays (ELISAs) were performed with standard methodologies (92) using strain RB51/SOD OMV for the coating antigen at a

concentration of 2.5 μg/ml in carbonate coating buffer. Mouse serum at a dilution of 1:100 in PBS from the vaccinated mice was used to bind to the coating antigen and secondary anti-mouse serum conjugated to HRP was used for color development. TMB membrane peroxidase substrate (KPL, Gaithersburg, MD) was added to the plate to cause color production and TMB stop solution (KPL, Gaithersburg, MD) was added to stop the reaction. The ELISA plates were read at 450nm and an OD values were obtained in a microplate reader (Versamax, Molecular Devices, Sunnyvale, CA).

Mixed Splenocyte culture

Cytokine assays were carried out as described previously (100). Briefly, two groups of three 6-week old female BALB/c mice were vaccinated i.p. with 15 μg of strain RB51 OMV or 15 μg of strain RB51/SOD OMV in 0.05 ml of saline and 0.05 ml (650 μg) of aluminum hydroxide adjuvant (Sigma-Aldrich, St. Louis, MO). As a negative control, three 6-week old female BALB/c mice were vaccinated with 0.1 ml of saline. Two weeks post-inoculation, each group was boosted with their corresponding vaccines in the same dosages. Five weeks post-boosting, the animals were killed by CO_2 asphyxiation and the spleens were harvested. Single-cell suspensions were prepared from the spleens of normal and vaccinated mice by placing the spleens on sterile 60-mesh stainless steel screens and mincing them with scissors. The splenocytes were cultured in 96-well plates at a concentration of 3.1×10^6 cells/well in the presence of either 15 μg of strain RB51 OMV, 15 μg of RB51/SOD OMV, 0.5 μg of concanavalin A (ConA), or no additives (unstimulated control). RPMI 1640 (Gibco-BRL, Carlsbad, CA) supplemented

with 2mM L-glutamine, 10% heat-inactivated fetal bovine serum, and 50 μM 2-mercaptoethanol was used to grow the cells for 5 days at 37^{o}C and 5% CO_2 atmosphere.

Cytokine Quantitation assays

After stimulation of splenocytes, supernatants of 5-day old cultures were tested for the presence of interferon-gamma (IFN-γ), interleukin-4 (IL-4), and interleukin-17 (IL-17) by solid phase sandwich ELISA. The ELISA kits were purchased from Cell Sciences (Canton, MA) and the assays were performed according to the manufacturer's instructions. The minimum detectable level for samples of IFN-γ, IL-4, and IL-17 was less than 15 pg/ml, less than 0.6 pg/ml, and less than 6 pg/ml, respectively.

Statistical Analysis

All data were analyzed by two-way analysis of variance using Prism software (GraphPad Software, La Jolla, CA). A *P* value of <0.05 was considered significant.

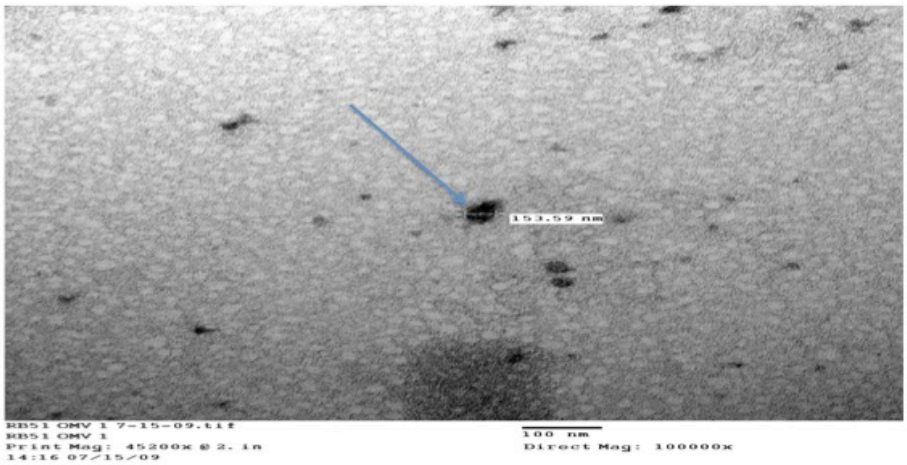
Results

OMV isolation and characterization

Rationale: OMV characterization was performed in order to visualize the OMV samples. The visualization ensured that the protein concentrations were due to actual, intact OMV.

OMV from strains RB51 and RB51/SOD were isolated by ultra-centrifugation of *Brucella* free culture medium and determined to be at concentrations of 8.1 mg/ml and 7.9 mg/ml protein, respectively. The samples were viewed by electron microscopy at 63,000x and 100,000x magnification and OMVs were found in all samples (Fig. 1).

A



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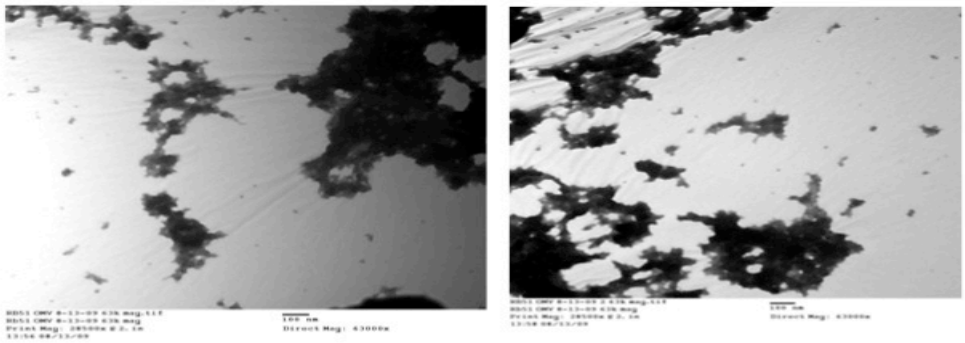


Figure 1: Electron microscopy of strain RB51 OMV. A) Electron microscopy at 100,000x magnification. The first panel shows the approximate size of a single OMV, previously shown in literature to range from 50 to 250 nm. The arrow is pointing to an individual OMV. B) Electron microscopy at 63,000x magnification. The second panel shows a large amount of OMV aggregated together.

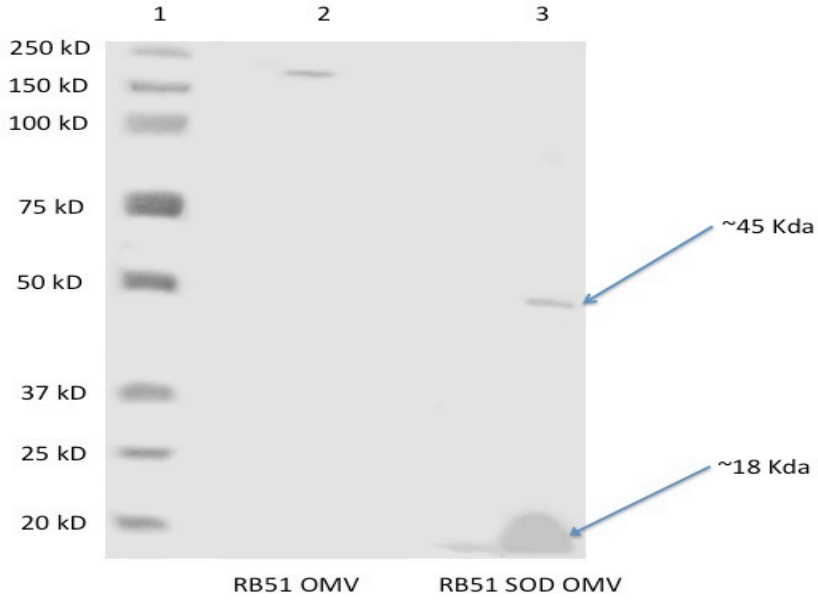
Immunodetection of Cu/Zn SOD in OMV from strain RB51

Rationale: In order to determine that strain RB51/SOD OMV contained higher levels of Cu/Zn SOD than strain RB51 OMV, immunoblot analysis was performed using a goat polyclonal anti-Cu/Zn SOD serum.

In the immunoblot, no 18 kDa band corresponding to Cu/Zn SOD protein (103) was detected in strain RB51 OMV (Fig. 2A). However, substantial levels of Cu/Zn SOD protein (~18 kDa) were detected in strain RB51/SOD OMV (Fig. 2A). In addition, an immunoreactive band was found at approximately 40-45 kDa that is roughly double the size of denatured Cu/Zn SOD, a phenomenon noted in previous literature (16, 66, 103).

The Coomassie blue stained SDS-PAGE gel showed strong bands at approximately 20 kDa likely to be attributed to Cu/Zn SOD. Strain RB51/SOD OMV exhibited bands at approximately 28 kDa and 31 kDa that likely correspond to outer membrane proteins. Strain RB51 OMV also exhibited a faint band at approximately 31 kDa that should correspond with an outer membrane protein (19, 24, 98). Strain RB51 OMV and strain RB51/SOD OMV exhibited different protein profile in the range of 50 kDa to 175 kDa (Fig. 2B).

A



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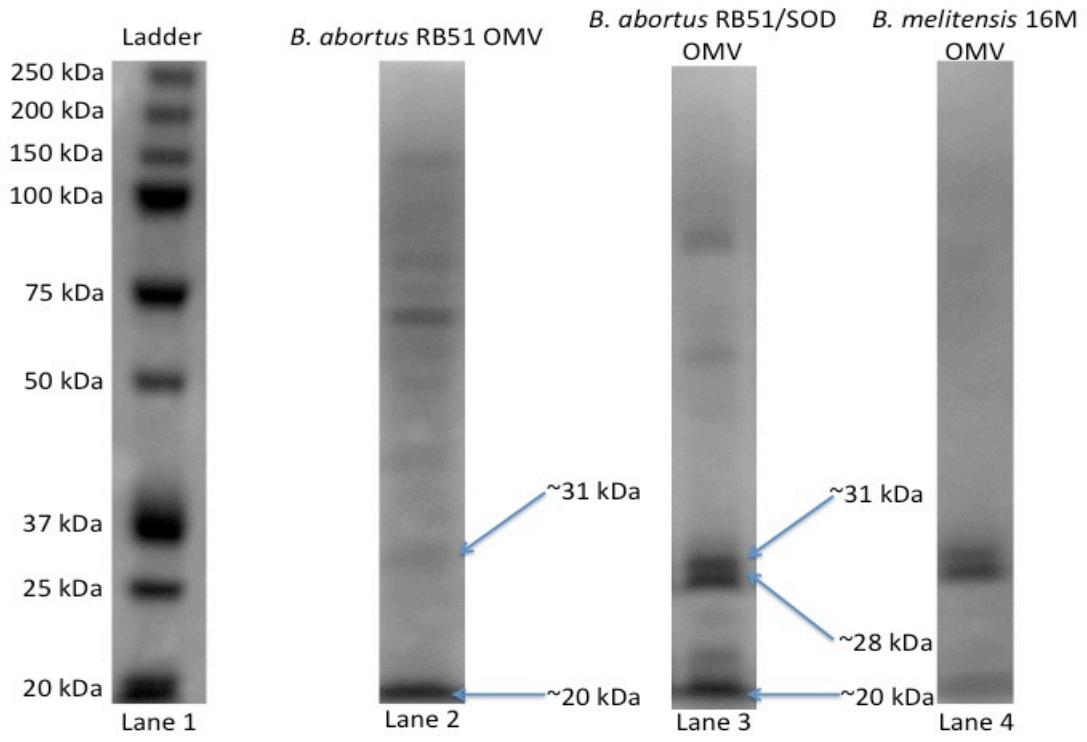


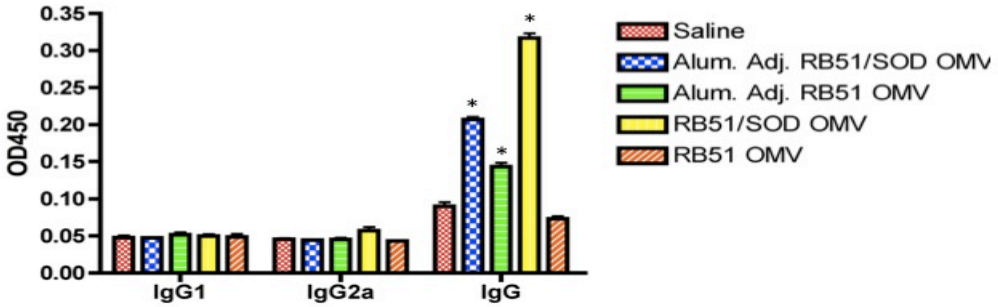
Figure 2. Western blot analysis of Cu/Zn SOD and Coomassie blue stained gels of OMV. A) The cellular extracts (150 μg) loaded are as follows: Lane 1, molecular mass standards; Lane 2, strain RB51 OMV; Lane 3, strain RB51/SOD OMV. Approximately 150 ug of OMV extract was loaded. The membrane was reacted with goat anti-Cu/Zn SOD serum and HRP-conjugated mouse anti-goat serum. B) Lane 1, molecular mass standards; Lane 2, strain RB51 OMV; Lane 3, strain RB51 SOD OMV; Lane 4, *B. melitensis* strain 16M OMV. The membrane was treated with Coomassie Blue stain overnight and destained the next day with acetic acid/methanol.

Serologic Response

Rationale: ELISA was performed on all of the serum samples to assess whether the mice vaccinated with strain RB51 OMV or strain RB51/SOD OMV preparations were developing a specific immunoglobulin response towards OMV.

As detected by ELISA, sera obtained from mice at 2 weeks post-vaccination contained no IgG1 or IgG2a antibody specific for strain RB51 OMV or strain RB51/SOD OMV, with or without adjuvant. However, there were significant amounts of OMV specific IgG antibodies developed in strain RB51/SOD OMV with aluminum adjuvant, strain RB51 OMV with aluminum adjuvant, and strain RB51/SOD OMV vaccinated mice (Fig. 3a). Sera obtained from mice vaccinated with different preparations of strain RB51 OMV at 2 weeks post-boost had significantly higher [$P < 0.05$] OD readings than mice vaccinated with saline only (Fig. 3b).

A



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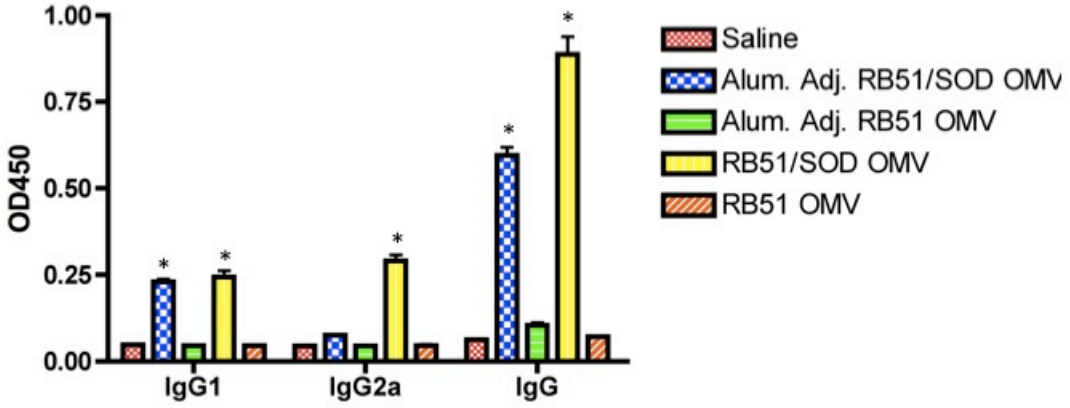


Figure 3. Relative serum antibody levels following immunization with strain RB51 OMV preparations. A) Serologic response 2 weeks following vaccination. B) Serologic response 4 weeks following boosting. The abbreviation Alum. Adj. refers to vaccine preparations that included aluminum hydroxide adjuvant. Significance was determined using two-way analysis of variance and a *P* value of ≤ 0.05 was considered significantly different than saline control and indicated by *.

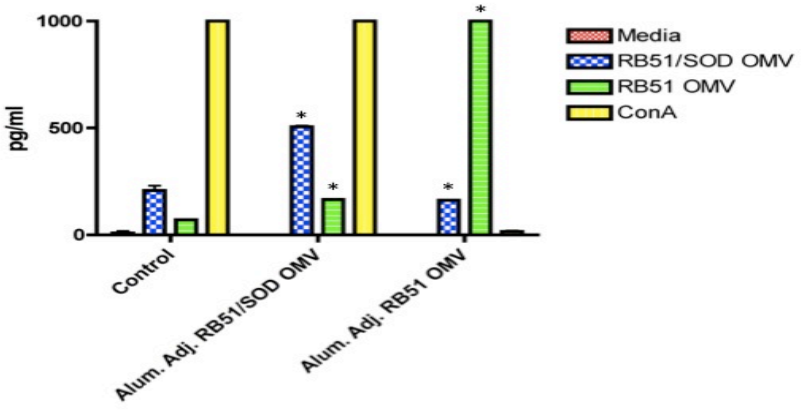
Cytokine Production in Mixed Splenocyte Cultures

Rationale: Cytokine production from mouse mixed-culture splenocyte cells was used to further characterize the specific immune response towards preparations of strain

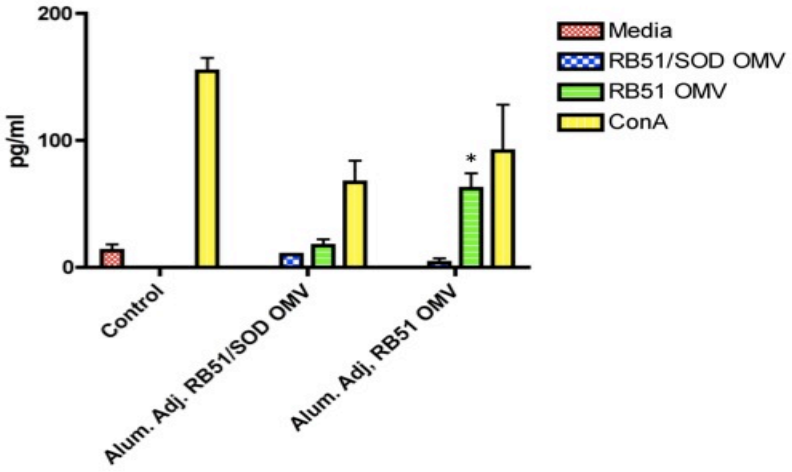
RB51 OMV vaccines. The cytokine response further clarifies the polarization of the immune system (i.e. Th1 vs Th2) caused by strain RB51 OMV.

Culture supernatants of immune splenocytes obtained from vaccinated mice at 5 weeks post-boosting produced significantly higher levels of IFN-γ in culture supernatants relative to saline vaccinated mice. The IFN-γ levels produced were considerably lower than from the splenocytes stimulated with ConA used as a positive control (Fig. 4a). Only culture supernatants from splenocytes of strain RB51 OMV vaccinated mice that had been stimulated with RB51 OMV showed significant levels of IL-4 production. The IL-4 level produced was similar to the level of IL-4 from ConA stimulated splenocytes (Fig. 4b). Vaccination down-regulated the amount of IL-4 that cells were able to produce when stimulated with ConA. Vaccination with strain RB51/SOD OMV with aluminum adjuvant down-regulated the amount of IL-17 that strain RB51 OMV stimulated cells were able to produce (Fig. 4c). Vaccination with strain RB51 OMV and strain RB51/SOD OMV both in aluminum adjuvant up-regulated the amount of IL-17 that cells were able to produce when stimulated with ConA.

A


1000
500
0
pg/ml
*
*
*
*
Media
RB51/SOD OMV
RB51 OMV
ConA
Control
Alum. Adj. RB51/SOD OMV
Alum. Adj. RB51 OMV

B


200
100
0
pg/ml
*
Media
RB51/SOD OMV
RB51 OMV
ConA
Control
Alum. Adj. RB51/SOD OMV
Alum. Adj. RB51 OMV

C

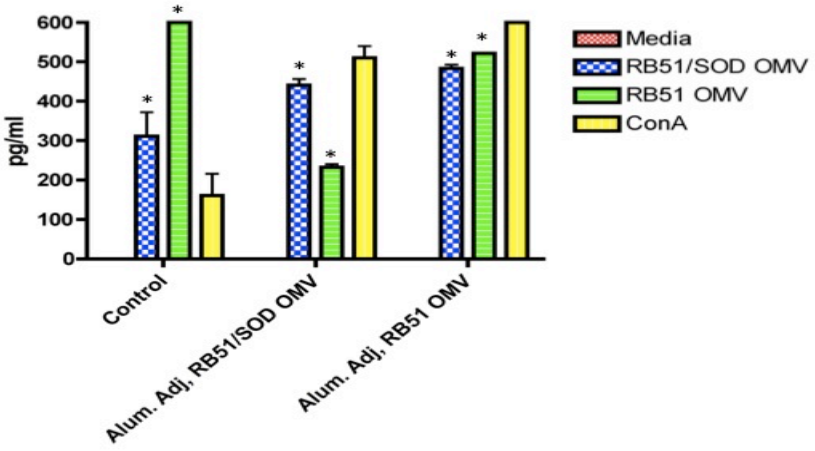


Figure 4. Cytokine responses to preparations of strain RB51 OMV in a mixed splenocyte culture derived from BALB/c mice 5 weeks post-boosting. The abbreviation Alum. Adj. refers to vaccine preparations that included aluminum hydroxide adjuvant. A) IFN-γ response to RB51 OMV stimulation. B) IL-4 response to RB51 OMV stimulation. C) IL-17 response to RB51 OMV stimulation. Significance was determined in all cases by two-way analysis of variance and a *P* value of ≤ 0.05 was considered significantly different than saline control and indicated by a *.

Measurement of *B. abortus* 2308 CFU in Spleens

Rationale: Protection experiments were performed to determine whether the level of immune response to the OMV was protective in a mouse model against brucellosis infection (68, 69, 72, 99).

The immunized groups of mice were challenged at 2 weeks post-booster vaccination with smooth, wild type *B. abortus* strain 2308. The splenic cfu in OMV vaccinated groups were approximately 0.1-0.5 log units lower than the unvaccinated controls. However, these differences were not statistically significant (Fig. 5).

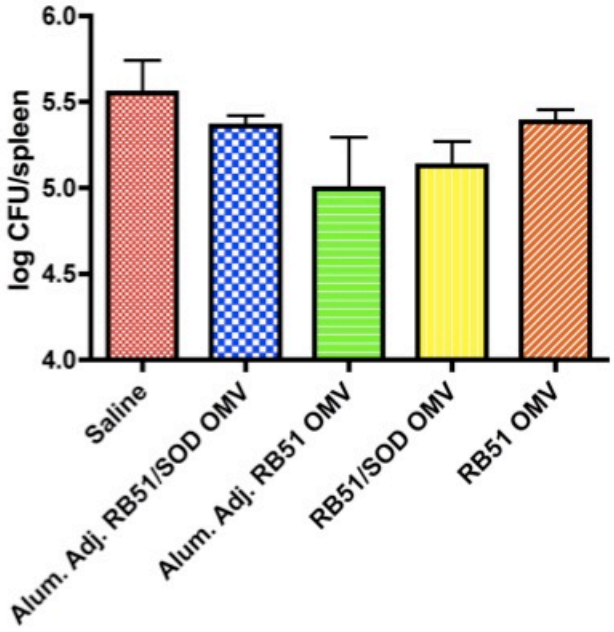


Figure 5. Splenic cfu from BALB/c mice challenged with *B. abortus* strain 2308. The differences between the groups were not statistically significant. The abbreviation Alum. Adj. refers to vaccine preps that included aluminum hydroxide adjuvant. Significance was determined by two-way analysis of variance and a P value of ≤ 0.05 was considered significantly different than saline control and indicated by a *.

Discussion

OMV based vaccines have been in use for many years. *Neisseria* spp. OMV were among the first human vaccines using OMV to provide protective immunity (63, 79). OMV vaccines have since been created for other pathogens including *Vibrio cholerae*, *Bordetella pertussis*, and *Salmonella enterica* serovar Typhimurium (5, 41, 42, 64, 77, 84, 85). These vaccines represent a unique method to stimulate protective immunity in a host. They are not live vaccines, so there is little to no risk of infection due to vaccination itself. However, OMV should afford better immunity than traditional subunit vaccines due to the presentation of the antigens in their same native forms as the live pathogen. The inability of OMV to cause infection makes OMV based vaccines very attractive for human and animal vaccination against *Brucella* spp. *Brucella* spp. infect over 500,000 humans annually, causing brucellosis and it is currently the most widespread zoonotic infection in the world (83). Despite these facts, there is currently no approved human vaccine against *Brucella* spp (83, 87). The work presented here suggests that *Brucella* spp. OMV can stimulate an immune response in the mouse model.

The electron microscopy images confirmed that OMV were present in the vaccines. At 100,000x magnification, individual OMV could be seen and fell within the typical size range as other, previously reported for isolated OMV (56). However, there was size disagreement between the scale present on the image and the size measured by a tool in the program that created the image. According to the measurement tool, the OMV were in the correct size range, at 153 nm, that has been previously reported (56). But the scale present on the image would place the OMV in the size range of 40 to 50 nm, which

is slightly smaller than the previously accepted range of 50 to 250 nm. This disagreement could be due to problems in the software and should be addressed when using this electron microscope to visualize OMV in the future. At 63,000x magnification and a higher concentration of OMV, clusters of OMV were visible and appear to have aggregated together (Fig. 1B). Working with lower dilutions, as in the 100,000x magnification image (Fig. 1A), allowed for the viewing of singular OMV.

Denaturing gels stained with Coomassie blue allowed for visualization of the protein profile present in the OMV. The *B. abortus* strain RB51/SOD OMV protein profile was compared to that from *B. melitensis* strain 16M OMV and *B. abortus* strain RB51. But the strain 16M OMV used were quite old and exposed to much freezing and thawing and the proteins had likely degraded. In fact, no protein bands were present in the strain VTRM1 OMV lane and that sample had likely completely degraded. Large bands were present around 20 kDa in the lane containing strain RB51/SOD OMV and *B. melitensis* strain 16M OMV lanes. These bands likely account for Cu/Zn SOD and strain RB51 OMV does have a small band at the same position. Other bands are present at approximately 25 kDa and 31 kDa in strain RB51/SOD OMV and likely account for outer membrane proteins while other proteins present in the OMV could have populated the periplasm (55). Strain RB51 OMV exhibited a single band at approximately 31 kDa that would likely be an outer membrane protein (19, 23). The protein profile of strain RB51 OMV slightly differed from that of strain RB51/SOD OMV in the range of 50-150 kDa. A proteomics study was not performed on strain RB51/SOD OMV and strain RB51 OMV, so the exact differences or reasons for differences cannot be elucidated. The SDS-

PAGE profile, along with the electron microscopy, further proves that the samples used for vaccination contained OMV from strain RB51 and strain RB51/SOD.

Immunoblotting was performed to ensure that strain RB51/SOD OMV did actually contain more Cu/Zn SOD than strain RB51 OMV. A large, immunoreactive band was present at approximately 20 kDa in the strain RB51/SOD OMV lane and was confirmed by immunoblotting using anti-Cu/Zn SOD serum (103). Strain RB51 OMV exhibited little to no immunologically detectable Cu/Zn SOD. The immunoblot definitively confirmed that strain RB51/SOD OMV contains a greater amount of Cu/Zn SOD than strain RB51 OMV.

Serology was performed to determine the type of antibody subclass response, i.e. IgG1 vs. IgG2a vs. IgG whole molecule, that strain RB51 OMV and strain RB51/SOD OMV caused *in vivo* (103). Mice immunized with strain RB51 OMV and adjuvant did show a significant increase in whole IgG level. But the increase was only seen at the first bleed, two weeks post-vaccination. The lack of response after the boost vaccination could be due to problems found in the concentration of OMV i.e. denaturing protein gels showed a much lower concentration of protein compared to the concentration estimated colorimetrically. However, the concentration problems were also present in strain RB51/SOD OMV vaccine, and that group of mice continued to produce antibodies according to the ELISA assays performed in this study. To ensure that concentration issues were not the reason behind the lack of response after boosting, the experiment would need to be repeated with accurate measures for concentration of OMV.

Strain RB51/SOD OMV caused a significant increase in IgG1, IgG2a, and IgG whole molecule titer levels at two weeks post-boosting. Increases in IgG1, IgG2a, and IgG whole molecule are indicative of a mixed response that could be eliciting both Th1 and Th2 responses towards the OMV. Polarization of the immune system, i.e. Th1 or Th2, was later determined by cytokine analysis. A mixed response skewed towards Th1 type immunity has been shown in other *Brucella* vaccine literature (47, 82, 101) using live, attenuated vaccines.

Strain RB51/SOD OMV in aluminum hydroxide adjuvant produced significant levels of IgG1 and IgG antibodies at two weeks post-boosting. Interestingly, the vaccine preparation produced little to no IgG2a, an immunoglobulin that is consistent with a Th1 polarized response (47, 101). Aluminum hydroxide adjuvant has previously been shown to skew immune responses to a Th2 bias (97). The use of aluminum hydroxide adjuvant could be very useful in the situation with a heterologous vaccine i.e. *Brucella* OMV over-expressing a heterologous antigen. If a Th2 polarization is necessary for protective immunity against a certain organism, e.g. flu virus, then OMV from strain RB51 over-producing an antigen from that target organism delivered with aluminum hydroxide adjuvant may be very effective. With the use of different adjuvants, an immune response could be "tailor made" towards a certain pathogen. Moreover, the use of adjuvants could really create wider uses for strain RB51 based OMV vaccines in the future.

Cytokine assays were performed to further elucidate the type of immune response that strain RB51 based OMV were inducing in the mice. The cytokines assayed were based on previous studies (37, 100), as they are indicative of Th1, Th2, or Th17 responses. IFN-γ was used to assess Th1 polarization, IL-4 was used to assess Th2 polarization, and IL-17 was used to assess Th17 polarization (37, 100).

Because a strong Th1 polarized response is necessary for the mice to clear *B. abortus* strain 2308 infection (87), IFN-γ was assayed. Strain RB51 OMV and strain RB51/SOD OMV stimulated splenocytes were able to generate significant increases in IFN-γ production when compared to negative controls. In previous studies, *B. abortus* extracts were shown to increase IFN-γ levels significantly (100). In the present study, strain RB51/SOD OMV increased IFN-γ to approximately half the level of the positive control. Strain RB51 OMV stimulation in IFN-γ production cannot be compared to the positive control in the same manner, because the ConA positive control did not produce levels of IFN-γ in this group. Lack of a positive control was likely due to human error and would have been repeated if splenocyte samples had been available to repeat the assay. Strain RB51/SOD OMV stimulated splenocytes showed higher levels of IFN-γ production than the *B. abortus* extract stimulated splenocytes when compared to positive controls (100). However, even with the increase in IFN-γ, protection was not achieved in this study.

Strain RB51 OMV vaccinated mice did not exhibit an immunoglobulin response indicating a Th1 response (i.e. IgG2a) that was specific to RB51 OMV (47); however,

they were able to induce a very strong IFN-γ response in primary, mixed splenocyte culture. The data indicate that even though strain RB51 OMV may not induce a strong immunoglobulin response, they do elicit a very strong cytokine based, Th1 directed response. Both strain RB51 OMV and strain RB51/SOD OMV stimulate a strong Th1 response that should help the host clear a *B. abortus* 2308 infection (72, 82, 104).

Strain RB51 OMV stimulated splenocytes produced significant levels of IL-4 indicative of a Th2 response (95). Strain RB51 OMV vaccinated mice showed no trend of decrease in splenic cfu following a challenge with smooth strain 2308. Strain RB51/SOD OMV stimulated splenocytes had little to no detectable levels of IL-4 and did provide protection against smooth strain 2308 in the mouse model.

IL-17 production, which induces a Th17 immune response, is a pro-inflammatory response that has been shown to be important in fighting infection in aged mice, allergy related disorders, and autoimmunity disorders (3, 37). Strain RB51 OMV and strain RB51/SOD OMV stimulated splenocytes showed significant increases in IL-17 production. In the unvaccinated control splenocytes there was a significant increase in the amount of IL-17 produced when stimulated with strain RB51 OMV or strain RB51/SOD OMV. However, vaccination with strain RB51/SOD OMV down-regulated the amount of IL-17 that strain RB51 OMV stimulated splenocytes were able to produce. Strain RB51 OMV vaccination did not have the same effect on strain RB51/SOD OMV stimulate splenocytes. Vaccination with either strain RB51 OMV with aluminum adjuvant or strain RB51/SOD OMV with aluminum adjuvant did not have a positive

effect on the amount of IL-17 produced when splenocytes were stimulated with strain RB51 OMV and strain RB51/SOD OMV. It would be interesting to repeat the experiment and stimulate with different purified outer membrane proteins and other purified proteins, such as Cu/Zn SOD, to determine which components of the OMV were causing this non-specific response. More importantly, it would be useful to determine if the OMV vaccines protect aged mice against a *Brucella* challenge (37) to determine if *Brucella* based OMV vaccines could potentially be effective in the elderly human population.

Clearance studies, as indicated by splenic CFU, suggested a protective trend in mice vaccinated with strain RB51 OMV in aluminum hydroxide adjuvant as well as in mice vaccinated with strain RB51/SOD OMV. However, neither of the decreases in splenic CFU was found to be statistically significant. Protection could potentially be achieved by the use of more OMV during vaccination or additional boosting. Alternatively, the route of immunization could be varied. In unpublished studies, OMV from *B. melitensis* given intramuscularly and boosted, induced protection in mice (A. Contreras, Mexico, personal communication).

The assay used to determine protein concentration (Bio-Rad, Hercules, CA) suggested a much higher concentration than what was observed after running an SDS-PAGE containing extracts of strain RB51 OMV or strain RB51/SOD OMV. Thus the small amount of actual OMV present in the vaccine preparations could account for the lack of protection observed in immunized mice. The amount of OMV delivered was

sufficient to produce an immune response in terms of higher amounts of serum immunoglobulins and cytokines, but not sufficient to produce a protective immune response (68, 82).

Repeating the protection studies with a known concentration of strain RB51 OMV and strain RB51/SOD OMV would be a very important next step. A more suitable assay to determine the protein concentration of OMV should be identified. Alternative routes of inoculation should also be considered as they could affect the type and quality of immune response that is stimulated (unpublished data, A. Contreras). In this study, intraperitoneal vaccination was chosen because of previously determined models for *Brucella* spp. vaccination and challenge protocols (72, 101, 103) using attenuated strains. However, strain RB51 OMV are not the same vaccine as a live, attenuated strain RB51 vaccine and may require a different route of administration in order to be effective.

In conclusion, the studies presented in this thesis indicated for the first time that *B. abortus* strain RB51 derived OMV are capable of producing an immune response in mice. A correlation existed between vaccination with strain RB51 derived OMV and a trends towards protection, although the protection levels were not significant. Future research should address repeating the protection studies with the aforementioned suggestions in order to determine the protective capabilities of strain RB51 derived OMV.

Tables

Table 1: List of vaccines used in the study presented in Chapter 2.

Vaccine	Description
Saline	Negative control for vaccination.
Saline/FRAP 20 ug	Determined immune response to native protein.
Freund's Adjuvant (Complete and Incomplete)/FRAP 20 ug	Standard characterized adjuvant to compare to irradiated strain RB51
Irradiated strain RB51 (10^6 and 10^4 cfu)/FRAP 20 ug	Determined if irradiated RB51 was a suitable adjuvant to induce a protective immune response towards FRAP.

Table 2: List of vaccines used in the study presented in Chapter 3.

Vaccine	Description
Saline	Negative control for vaccination.
15 ug RB51 SOD OMV in Aluminum Hydroxide Adjuvant (Alum. Adj. RB51 SOD OMV)	RB51 OMV that over express Cu/Zn SOD exported to the periplasm. This vaccine was based in adjuvant to increase antigenicity.
15 ug RB51 OMV in Aluminum Hydroxide Adjuvant (Alum. Adj. RB51 OMV)	RB51 OMV isolated from normal *B. abortus* RB51. This vaccine was based in adjuvant to increase antigenicity.
15 ug RB51 SOD OMV (RB51 SOD OMV)	RB51 OMV that over express Cu/Zn SOD exported to the periplasm.
15 ug RB51 OMV (RB51 OMV)	RB51 OMV isolated from vaccine strain *B. abortus* RB51.

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