

**ASSESSMENT OF THE EXPRESSION
OF *BRUCELLA ABORTUS* HEAT SHOCK PROTEIN, GROEL, IN
VACCINIA VIRUS TO INDUCE PROTECTION AGAINST A
BRUCELLA CHALLENGE IN BALB/C MICE**

by

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(ABSTRACT)

B. abortus is an intracellular facultative bacterial pathogen which causes abortion in cattle and undulant fever in humans. Cattle vaccines such as *B. abortus* strains 19 and RB51 are live vaccine strains which protect approximately 75% of the vaccinated animals. No effective vaccines are available for the prevention of brucellosis in humans. We are developing vaccinia virus recombinants expressing various *B. abortus* proteins to prevent brucellosis in susceptible mammalian species. In this work the *B. abortus groEL* gene encoding the antigenic heat shock protein GroEL was subcloned into vaccinia virus via homologous recombination. Expression of the GroEL protein in vaccinia infected cells *in-vivo* was confirmed by immunoblotting. Groups of 5 female BALB/C mice were injected with the vaccinia recombinant or appropriate positive and negative control vaccines. Mice were bled and their humoral immune responses assessed. In addition, mice were challenged with virulent *B. abortus* strain 2308 and protection measured by the rate of splenic clearance of live *Brucella*. In spite of demonstrating specific GroEL antibodies in recombinant vaccinia injected mice, no significant level of protection was demonstrable. Preliminary lymphocyte transformation assays were carried out to establish if a cell mediated immune response to GroEL was induced in the vaccinated animals.

This thesis is dedicated to
my husband, Erkan
with love and gratitude

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List of Abbreviations:

LPS	= lipopolysaccharide
ATP	= adenosinetriphosphate
Hsp(s)	= heat shock protein(s)
kb	= kilobase pair
TK	= thymidine kinase
DNA	= deoxyribonucleic acid
kDa	= kilodalton
RNA	= ribonucleic acid
HuTK	= human thymidine kinase
CPE	= cytopathic effect
ml	= milliliter
μl	= microliter
mg	= milligram
μg	= microgram
cm	= centimeter
BdUR	= bromo-deoxyuridine
pfu	= plaque forming units
min	= minute
cfu	= colony forming units
MOI	= multiplicity of infection
g	= gram
PBS	= phosphate buffered saline
mM	= millimolar
ID	=intradermal
IP	= intraperitoneal
IPTG	= isopropyl-beta-thiogalactopyranoside
EDTA	= ethylenediaminetetraaceticacid
SDS-PAGE	= sodium dodecylsulfate polyacrylamide gel electrophoresis
DTH	= delayed type hypersensitivity
LTA	= lymphocyte transformation assay
CMI	= cell mediated immunity
MBP	= maltose binding protein
FBS or FCS	= fetal bovine (calf) serum
EMEM	= Eagle's minimum essential medium
RPMI	= Roswell Park Memorial Institute

Chapter 1

Introduction:

Brucellosis is a chronic infection that is characterized by abortion and infertility in animals and undulant fever in humans (1). The members of Brucellae responsible for this disease are highly infectious and can infect a variety of wild and domestic animals. The mode of the infection is via the mucosal membranes of the digestive tract, genital tract and skin. The bacterial pathogen passes from the point of entry via the lymphatics to the regional lymph nodes and, after multiplication, to the thoracic duct and then to the parenchymatous organs and other tissues (9). The pathogen usually localizes in the organs of the genital tract due to a growth stimulating polyhydric alcohol erythritol in this organ system and causes clinical signs such as abortion, still births, epididymitis and infertility in domestic and wild animals. The pathogen can also localize in the liver, lungs, lymph nodes or spleen where it produces granulomatous foci. The disease in humans known as undulant fever can be treated with antibiotics such as streptomycin and tetracycline. Treatment of the chronic form of brucellosis is not always successful.

The bacteria in the genus *Brucella* are small gram negative, non-motile coccobacilli. The six species of the genera share more than 90% DNA homology. *B. abortus*, *B. melitensis* and *B. suis* occur in the smooth virulent phase and they are responsible for the naturally occurring cases of brucellosis in cattle, goats, swine and along with *B. canis* which is the causative agent of canine brucellosis can also cause disease in humans (1). The *Brucella* species have no capsular material, fimbriae or pili. The lipopolysaccharides (LPS) and outer membrane proteins (12, 13) are part of the virulence factors of the smooth (virulent) strains. A homopolymer of perosamine known as the O-side chain component of the LPS (8) appears to be an immunodominant antigen. Like many other gram negative bacteria, *Brucella* show smooth to rough colonial variation. The change of smooth to rough is associated with loss of virulence in some animal species and tendency to agglutinate. All *Brucella* species are closely related and most smooth strains share two O-side chain antigens which are designated A and M (8).

Brucella can reside and multiply within macrophages and as such, establishment of a chronic infection depends on the ability of the *Brucella* to survive intracellularly (17, 40). Therefore, to obtain protection it is essential to reduce the intracellular survival of *Brucella*. Activation of macrophages by interferon-gamma leads to destruction of *Brucella* (6). This suggests that T cells responsible for interferon-gamma production are of major importance in any immunity developed against brucellosis.

In veterinary medicine, protective immunity to brucellosis can be induced by attenuated live vaccines in cattle like *B. abortus* strain 19 (13). The humoral immune response of the host infected with smooth *Brucella* strains is predominantly directed against the O-side chain

component of LPS (12). Live attenuated vaccine strain 19 induces antibodies which interfere with the serological tests, can induce abortions in pregnant animals and provides only about 65-75% protection (10,34), therefore, it is not considered as an ideal vaccine. Other vaccines such as killed rough *B. abortus* strain 45/20, requires the use of an adjuvant and booster immunizations; overall, results of vaccine trials with 45/20 strain in cattle have not been satisfactory (28). *B. abortus* strain RB51 which is deficient in O-side chain has become the official vaccine strain in United States to prevent brucellosis in cattle (since March, 1996). This strain is stable both *in-vivo* and *in-vitro*, shows reduced virulence, induces cell mediated immunity and also elicits antibodies specific for a limited number *B. abortus* antigens excluding O-side chain (41). As of June, 1997 more than 5 million calves have been vaccinated with *B. abortus* vaccine strain RB51(35, Colorado Serum Company, personal communication through Dr. G. G. Schurig). Although strain RB51 has been approved as a vaccine for cattle vaccination, it is unlikely to be used in humans because the nature of the mutations causing the rough phenotype are undefined. The transfer of T-cells from strain RB51 immunized mice confers protective immunity against a virulent challenge (3) while transfer of antibodies does not (6); this suggests that immunity to brucellosis requires a strong cell mediated immune (CMI) response (4). Two types of live *Brucella* vaccines are being developed using recombinant DNA technology. In one type of vaccine, specific *Brucella* genes have been identified, which when mutated make the strain unable to synthesize purines or produce O-side chain (11). These mutant strains are attenuated and currently undergoing testing for their ability to induce protection. In the second type of vaccine, specific *Brucella* proteins are being tested *in-vitro* for their ability to elicit cell mediated immune mechanisms (DoD Grant 4-30565). Once these putatively protective proteins are identified, they will be delivered by a live host/vector and tested for their ability to induce protection. Knowing that *Brucella* can localize within the macrophages, it is important for a vaccine to enhance the nonspecific bactericidal activity of the macrophages. There is not much information about the proteins that are required for the survival, growth and pathogenesis of *B. abortus* in the intracellular environment of phagocytic cells. However, evidence from other bacterial systems indicates the expression of bacterial genes is coordinately regulated in response to environmental changes (30). These include genes that encode heat shock proteins (Hsp) which are produced particularly in response to stress conditions. Studies show that the GroEL protein, a heat shock protein and a chaperonin, is synthesized following phagocytosis of *Brucella* by macrophages (25). Moreover, additional evidence suggests Hsps are major antigens involved in the T-lymphocyte activation and induction of protective immunity (23, 29, 42).

The chaperonins GroEL and GroES belong to ubiquitous class of related chaperone molecules in mitochondria, chloroplasts and bacteria (5). The main function of these proteins is to prevent incorrect interactions within or between non-native polypeptide chains. Chaperonins are large cylindrical complexes composed of a pair of stacked rings with seven to nine subunits each (19). Each of the monomeric units of GroEL is composed of an apical, an intermediate and an equilateral domain. The equilateral domain is the largest one and provides the majority of

subunit to subunit interactions within the heptameric rings and all interactions across the equilateral plane of the cylinder; this domain also contains an ATP binding site. The intermediate domain is the smallest of the three domains and serves as the hinge, whereas the apical domain forms the opening of the central channel (20). For many proteins, completion of folding requires interaction with the oligomeric ring-shaped chaperonin. Under most conditions a single GroES ring binds to one end of the GroEL double ring exerting a negative cooperative effect on the opposite GroEL ring that reduces the affinity for binding of a second GroES (24). GroES causes an increase in the cooperativity of the ATPase activity of GroEL. These proteins bind the partially folded polypeptide in their central cavity and promote folding by ATP-dependent cycles of release and rebinding. In these reactions, these chaperones interact predominantly with the hydrophobic surfaces exposed on nonnative polypeptides thereby preventing incorrect folding and aggregation (20). Chaperones interact with a large number of different substrate polypeptides and do not possess steric information that would interfere with the folding of the protein. They prevent unproductive intra- and intermolecular interactions and enable the unfolded peptide to reach the defined structure specified by its amino acid sequence (18).

The heat shock response is a widespread phenomenon found in all living cells. Proteins associated with this response have been found to be highly conserved. Studies on *Brucella groEL* and *groES* genes show that they are adjacent on one of the two *Brucella* chromosomes and comprise a functional operon (16). In spite of the evolutionary distance between *Escherichia coli* and *B. abortus*, their hsp genes share about 60 % sequence identity.

Due to the immunogenic characteristics of heat shock proteins (23, 29, 42) and their ability to stimulate cell mediated and humoral responses, the genes encoding Hsps are thought to be good candidates for the generation of a new recombinant vaccine against brucellosis. In addition because *Brucella* is an intracellular pathogen, preparing a recombinant vaccine that induces cell mediated immunity would make it more efficient as a vaccine. Studies show that vaccinia virus expressing *Brucella* antigens can induce antibodies against both vaccinia virus and a *Brucella* HSP protein Htr A (44).

Vaccinia virus belongs to the *Orthopox* genus of the *Poxviridae* family. *Orthopox* viruses are DNA viruses which are unique in their ability to replicate within the cytoplasm of infected cells. They have a double stranded DNA genome about 180-220 kb long and have hairpin loops at each end (38). These viruses contain all the genes needed for the synthesis of virus macromolecules and replicate cytoplasmically. Numerous virus encoded enzymes, including a multisubunit of DNA dependent RNA polymerase, transcription factor, methylating and capping enzymes and poly (A) polymerase, are packed within the virus core and enable viruses to synthesize translatable mRNAs with typical eukaryotic features after entry into a cell (32). Virus assembly occurs in specialized areas of the cytoplasm; mature particles move to the cell periphery and become wrapped in the Golgi body, producing intact viral progeny.

Vaccinia virus has long been known as an efficient vaccine against smallpox. This virus had been used in the eradication program of smallpox due to cross reactivity with *Orthopox* viruses. In 1980, the Assembly of the World Health Organization declared that smallpox was eradicated and recommended the discontinuation of smallpox vaccination. Coincidentally, 1980 was the first year in which vaccinia virus was used as an expression vector (32). Aside from the efficiency of vaccinia virus replication within the cytoplasm, it has several characteristics which make it a very effective tool in recombinant vaccine production. The DNA genome of the virus can accept up to 25 kb foreign DNA without much change in the infectivity and functional characteristics of the virus (43). Certain genes within the genome, such as thymidine kinase (TK) (26) and vaccinia growth factor (46), permit the generation of recombinants by simple cloning techniques. Deletions within the genome, up to 18 open reading frames encoding functions implicated in the pathogenicity, result in the less virulent vaccinia strain NYVAC (37). Cytoplasmic replication of the virus eliminates special requirements for nuclear processing and transport of RNA (32). Moreover, vaccinia DNA contains strong promoters not recognized by eukaryotic transcription machinery. This enables high expression of a desired protein encoded by a foreign gene without any interruption from the eukaryotic host. Another reason for the popularity and diverse use of vaccinia virus is its wide host range and ability to integrate and package large amounts of DNA without loss of infectivity (31).

Recombinant vaccinia viruses induce both humoral and cellular immune responses (7,14,36). The induction of a strong major histocompatibility complex class I cytotoxic T-cell response is a major advantage of infectious recombinant vaccinia virus compared to inactivated or subunit vaccines (33,45). Moreover, a number of studies demonstrated that expressing a single antigen from specific bacterial pathogens in vaccinia virus produces cell mediated and humoral immune responses in animal models (2,15,21,22,27). Additionally, our studies with the *Brucella* antigens HtrA (44), GroEL, and the *Brucella* 18 kDa protein (unpublished observations) showed that *Brucella* antigens can be successfully expressed in vaccinia virus. Bacterial heat shock proteins have been shown to be important immunogens capable of stimulating both T cells and B cells. Experiments with different bacterial GroELs from *Legionella pneumophila*, *E. coli*, *Mycobacterium leprae* and *M. tuberculosis* have shown that these proteins induce cell-mediated and humoral immune response and increase cytokine levels (39).

This study has been done in order to achieve our long term goal of preparing a live vaccinia virus recombinant vaccine against brucellosis. The specific objectives of this thesis are to:

- a. develop a recombinant vaccinia virus expressing the *Brucella abortus* groEL gene.
- b. use this recombinant in a mouse model to assess induction of humoral and cell mediated and humoral immunity.

- c. determine whether the use of recombinant protects mice against a virulent *Brucella* challenge

Chapter 2

Materials and Methods

Construction of *Brucella abortus* GroEL/Vaccinia Virus Recombinants

In order to prepare the vaccinia virus expression vector coexpressing *B. abortus* GroEL heat shock protein, plasmid pBA2168 (M. Roop et al., 1992) was digested with restriction enzymes *EcoR* I, *Sal* I and *Dra* II (Promega, Pittsburgh, PA). Overhanging ends were filled in using Sequenase enzyme (United States Biochemical [USB], Cleveland, OH). The resulting 1.7 kb fragment containing the *B. abortus groEL* gene was purified from 1 % agarose gel using Wizard™ purification columns (Promega, Pittsburg, PA); the fragment was ligated into the *Sma* I site of shuttle plasmid pSC11 (Chakrabarti et al., 1985) to produce plasmid pSBGroEL. The correct orientation of the gene within plasmid pSBGroEL was confirmed by restriction enzyme digestion and gel electrophoresis. Human thymidine kinase deficient 143B cells (HuTK⁻ cells) (American Type Culture Collection, ATCC, Rockville, MD) were grown to 80% confluency in Eagle's Minimum Essential Medium (EMEM) (ICN, Costa Mesa, CA) containing 5% fetal bovine serum (FBS) (Intergen, Purchase, NY) in 25 cm² flasks, infected with vaccinia virus strain Western Reserve (WR) (ATCC) at a multiplicity of infection (MOI) of 0.05 and incubated for 2 hours at 37⁰C, in a 5% CO₂ incubator. One µg of pSBGroEL was dissolved in 50 µl of sterile distilled water and mixed with 50µl undiluted lipofectin reagent (Gibco-BRL, Grand Island, NY) and incubated for 25 minutes at room temperature. This DNA/lipofectin mixture (100µl) was added to 1 ml of EMEM and added to WR infected HuTK⁻ cells at 80% confluency. The infected cells were then supplemented with 3.0 ml of (EMEM). After a 4+ cytopathic effect (CPE) had developed (usually in 48-72 hours), the cells were ruptured by 3 cycles of freezing in liquid nitrogen and thawing at 37⁰ C. The cell lysates, containing the putative recombinant virions, were serially diluted in 10-fold steps and subcultured onto a new monolayer of HuTK⁻ cells in flat-bottom six-well plate (about 9.6 cm² surface area) (Falcon™, Franklin Lakes, NJ) with EMEM containing 25 µg of bromodeoxyuridine (BdUR) per ml for selection of recombinant virus. Following a 4+ CPE development, the medium was aspirated and the infected cells were overlaid with 1 ml of plaquing media (2x EMEM with 50µg BdUR) containing 0.6 mg/ml of Blueo-gal (Gibco-BRL, Grand Island, NY). Blue plaques, produced by replicating recombinant virions expressing the *lacZ* gene, were collected and used to enhance the virus content of the plaques by inoculating a confluent layer of HuTK⁻ cells in either 25 cm² flasks or six well tissue culture plates. Replication of the recombinant virus was assessed by CPE and the presence of blue plaques in the cell monolayer. Recombinant virus was harvested, plaque purified and enhanced by infecting larger volumes of cell monolayers (in a 25 cm² flask) two more times to develop the recombinant virus designated as WRSBGroEL. Same experiments were also carried out for to generate the vaccinia virus recombinant WRSC11 with shuttle vector pSC11.

Recombinant virus was tested for infectivity, purity and ability to induce GroEL antibodies in mice. 10^4 plaque forming units (pfu) of recombinant virus were injected into each of three BALB/C mice. One of the mice was killed six days later and the recombinant virus was isolated from ovaries and tested for CPE and β -galactosidase activity in HuTK⁻ cells. The second mouse was killed on day thirteen post infection and the same procedures were followed in order to isolate and to test the infectivity of the recombinant virus in the cell culture. The third mouse was kept alive to obtain serum for serological tests. After isolating the virus from the second mouse, the recombinant virus was plaque purified again and enhanced three more times in order to reach 10^7 pfu/ml, the desired infective dose for mice vaccination. The high titer recombinant was used to make WRSBGroEL virus stock by infecting HuTK⁻ cells in a 75 cm² flask at a MOI of 1. After a 4+ CPE was observed, the content of the flask was aspirated and centrifuged at 1000g for 5 min. The pellet was saved and resuspended in 1ml of minimal cell culture medium OptiMem (Gibco-BRL, Grand Island, NY). The virus was released from the cells by three consecutive freeze-thaw cycles.

Protein Expression Analysis

To screen for *Brucella* GroEL expression, 90% confluent HuTK⁻ cells in 75 cm² flasks were infected with recombinant vaccinia virus at an MOI of 10. After an incubation of 24 hours at 37⁰ C, the culture medium was aspirated and saved. The cells were separated from the flask by adding 0.5ml of 2x trypsin (Gibco-BRL, Grand Island, NY) into the flask and rocking it until all the cells were released. The cells were added to the saved medium, pelleted by centrifugation for 5 min at 1000g, washed with 10ml of 0.1M PBS twice, resuspended in a final volume of 200 μ l of PBS and heated to 95⁰ C for 5 min. to kill the virus. Sodium dodecyl sulfate was added to 0.2 %, the suspension vortexed, heated again for 2 min and centrifuged at 1500g, the supernatant was recovered and the pellet discarded. Samples were analyzed for protein expression by separation in 10 % SDS-PAGE as defined previously, followed by transfer to nitrocellulose (0.45 micron Nitropure membrane) (MSI Filters, Westboro, MA); the membrane was probed with serum from a goat hyper-immunized with killed strain RB51 (No. 48) as primary antibody and rabbit anti-goat IgG coupled to hydrogen peroxidase as secondary antibody. The serological reactions were visualized by incubation in a developing solution consisting of 0.060g 4-chloro-1-naphthol in 20ml methanol added to 100 ml of Tris buffered saline (TBS)(0.15M NaCl, 20mM Tris, pH 7.5) with 36 μ l of 50 % hydrogen peroxide. The development of the color reaction was stopped after 5 min by placing the membrane in distilled water.

Immunization

Five groups of 8 female BALB/C mice (Charles River Laboratories, Wilmington, MA) each were used. The first and second groups were injected with 10^7 pfu of WRSBGroEL intradermally (ID) and intraperitoneally (IP) respectively. The third group was inoculated IP with 10^7 pfu/ml of vaccinia virus/shuttle plasmid recombinant WRSC11. The fourth group received 2.6×10^8 colony forming units (cfu) of *B. abortus* rough strain RB51 (IP) as a positive control for protection and the fifth group received saline IP.

GroEL Antigen Preparation to Use in Cell Mediated Immune Response Analysis

A 1.57 kb *Brucella groEL* gene missing 130 base pairs from the 3 prime end was subcloned into *BamH* I and *Pst* I site of the pMALTM-c2 expression plasmid (New England Biolabs (NEB), Beverly, MA). The *groEL* gene was ligated to the *mal* E gene encoding for maltose-binding protein (MBP) to create a *malE::groEL* fusion gene. *Escherichia coli* DH 5 alpha cells were transformed with this plasmid and grown to 2×10^8 cells/ml in glucose enriched broth (10g tryptone, 5g yeast extract, 5g NaCl, 2g glucose). The 150ml culture was induced to produce the fusion protein by addition of isopropyl-beta-thiogalactopyranoside (IPTG) to produce the fusion protein 0.3mM for two hours to produce MBP::GroEL. The cells were harvested by centrifugation at 4000g for 20 min and resuspended in 5ml of column buffer (20 mM Tris-HCl pH 7.4, 200mM NaCl, 1mM EDTA). The suspension was sonicated 8 times for 15s each at 35% efficiency to lyse the cells; the extract was centrifuged at 9000g for 30 min, the supernatant (crude extract) saved, run through a 7ml amylose resin column (NEB, Beverly, MA) and bound fusion protein was eluted with 20ml of 10mM maltose. Eluted fusion protein was then concentrated to 0.6mg/ml by three consecutive filtration steps using polysulfone 10 Ultrafuge filters (MSI filters, Westboro, MA).

Immunological Tests and Assays

Delayed Type Hypersensitivity Test (DTH): Three mice from each vaccinated group were tested for DTH reactions in the footpad. The left foot of each mice was injected with 40µg of purified MalE::GroEL fusion protein and the right foot of each was injected with 30µg crude extract containing the Maltose binding protein only. The swelling reaction was measured in millimeter on each footpad (for each mouse) after 48 hours.

Humoral Immune Response Analysis: Either one or two mice from each group were bled intraorbitally at the third, fifth, seventh, ninth and twelfth weeks post immunization. The sera was diluted 1:50 and analyzed for GroEL antibodies by a Western Blot analysis using the following antigen preparations: WRSBGroEL, WRSC11 and *B. abortus* RB51 whole cells. Goat anti-RB51 (No.48) serum was used as a positive control.

Lymphocyte Transformation Assays: Three mice from each group (the same as used in the DTH test) were killed by CO₂ inhalation and their spleens removed. Single-cell suspensions were prepared by pressing the whole spleen tissue through a stainless steel sieve (mesh size 60, Sigma-Aldrich, St. Louis, MO). The cell suspensions were washed three times with 10ml RPMI medium (Sigma-Aldrich, St. Louis, MO), red blood cells were lysed with lysis buffer (0.1M NH₄Cl, 0.1M EDTA, 1M KHCO₃) and resuspended in 20ml of complete tissue culture medium (CRPMI), comprised of RPMI medium, 5% FBS, l-glutamine and 50 μM 2-mercaptoethanol. Viable cell counts were determined by mixing an equal volume of cell suspension with trypan Blue (Sigma-Aldrich, St. Louis, MO) and counting in a hemocytometer. Cells were adjusted to 5x10⁶ viable cells/ml and 100μl of cells dispensed in triplicate with 100μl antigen in round bottom 96-well tissue culture plates. Column purified MalE::GroEL fusion protein (10μg/ml) was used as the experimental antigen, concanavalin A (Sigma-Aldrich, St. Louis, MO) (1μg/ml) as a positive lymphocyte replication control and strain RB51 (10μg/ml) cell extract as a positive *Brucella* antigen reaction. After 48 hours incubation at 37° C in a 5% CO₂ incubator, the cells were pulsed for 18 hours with 0.5μCi (20μl of 1:20 dilution with CRPMI) of tritiated thymidine (thymidine, [methyl-³H], 6.7Ci/mmole) (ICN, Costa Mesa, CA) with specific activity 6.7Ci/mmole. Cells were harvested onto glass microfiber filters GF/A (Whatman Lab Hillsboro, OR) and washed with distilled water; insoluble tritiated thymidine counts were determined by liquid scintillation spectrometry (Beckmann LS810, Schaumburg, IL) and results are expressed as a stimulation index (SI). The SI is calculated by:

$$\frac{\text{insoluble}[H^3] \text{ thymidine cpm of spleen cells exposed to antigen}}{\text{insoluble } [H^3]\text{thymidine cpm of spleen cells unexposed to antigen}}$$

Protection Analysis

At eight weeks post-immunization five mice from each group were challenged IP with 2.2x10⁴ cfu of *B. abortus* smooth strain 2308. The mice were killed after 14 days, their spleens removed and macerated by grinding them in 1ml Trypticase Soy TM broth (TSB, Becton Dickinson, Cockeysville, MD) containing sterile sand using a Teflon coated pestle. The suspensions were serially diluted in ten fold dilutions in TSB and five drops of 10μl each of each dilution was plated out on Trypticase Soy Agar (TSA). The plates were incubated for four days at 37⁰C, 5% CO₂ incubator; colony counts obtained and the average *B. abortus* 2308 cfu per mouse spleen for each group was determined. The results were analyzed by paired t-Test (Sigma Plot TM, Scientific Graphic Software version 2.0, Jandel Scientific, San Rafael, CA) by comparing each group to the negative control i.e., saline group.

Chapter 3

Results

Vaccinia Virus Recombinants Expressing *Brucella groEL* Gene

Subcloning of the *B. abortus groEL* gene into shuttle vector pSC11 is illustrated in Fig 1. This recombinant plasmid, pSBGroEL, contains a moderate-strength compound early/late promoter, p7.5, which expresses the *B. abortus groEL* gene; a late vaccinia virus promoter, p11, is used to express the *E. coli lacZ* gene. The expression cassette (p7.5 and p11) is flanked by segments of the vaccinia virus *TK* gene which permits homologous recombination with the wild type vaccinia virus genome. A double cross-over event occurs between homologous regions of vaccinia virus DNA sequences TK_L and TK_R and generates *B. abortus groEL*/vaccinia virus recombinants.

A *B. abortus groEL*/vaccinia virus recombinant WRSBGroEL was obtained by using thymidine kinase selection and β -galactosidase screening (See Materials and Methods). The level of infection and color development were assessed using an inverted light microscope. The characteristic vaccinia virus cytopathic effect on fibroblastic cells, rounding up and sloughing off from the bottom of the tissue culture flask, is shown in Fig 2. The recombinant virus was then subjected to several rounds of plaque purification in which the blue plaques were selected to ensure the absence of residual wild-type virus (Fig 3).

The expression of *B. abortus* GroEL protein in a selected vaccinia virus recombinant was demonstrated by a Western Blot analysis (Fig 4). An *E. coli* adsorbed goat anti-*B. abortus* strain RB51 hyperimmune serum (No. 48, Schurig et al., 1991) reacted only with GroEL protein produced by WRSBGroEL (Fig 4, lane 2). Proteins expressed by *B. abortus* RB51 show a spectrum of proteins recognized by goat No. 48 antiserum. The virus WRSBGroEL was tested in three mice for infectivity, purified and enhanced to 10⁷pfu/ml. Several recombinant virus stocks were analyzed for GroEL expression in a Western Blot utilizing goat No. 48 hyperimmune serum (Fig 5). *Brucella* GroEL antigen expression by WRSBGroEL isolated from vaccinated mice was also analyzed with a Western Blot (Fig 6). Mouse sera, obtained following the third week of vaccination with WRSBGroEL was used to probe a membrane containing this isolate and other specific antigen preparations. The sera reacted only with GroEL in *B. abortus* RB51 preparation (Fig 6, lane 3). GroEL antigen and a spectrum of vaccinia virus proteins were recognized in different WRSBGroEL stocks prepared either prior or after the recombinant was isolated from mice (Fig 6, lanes 4,5,6). Only vaccinia virus proteins were recognized in WRSC11 (Fig 6, lane 8) and a spectrum of background proteins were recognized in HuTK⁻ cell protein preparation (Fig 6, lane 7).

Humoral Immune Response Analysis of Vaccinated Mice:

The safety of the WRSBGroEL recombinant was tested in a preliminary trial by inoculation of 6 (>8 months old) mice with 10^4 , 10^6 and 10^7 pfu doses (two for each). All of the six mice survived and no pox virus lesions were observed on their skins. Sera from these mice were obtained in the third week post inoculation and used to develop a Western Blot to compare the humoral response of the vaccinated animal at each dose (Fig 7). All of the sera from animals vaccinated with either 10^4 , 10^6 or 10^7 pfu of recombinant virus were able to recognize GroEL antigen in a strain RB51 antigenic preparation (Fig 7, lane 1 in each membrane); a spectrum of vaccinia virus proteins and *B. abortus* GroEL in WRSBGroEL antigen lanes (Fig 7, lane 2 in each membrane) and only vaccinia virus proteins (Fig 7, lane 3) in the plasmid control vaccinia virus recombinant, WRSC11, were recognized. All mice responded to the GroEL antigen and there was not a significant dose effect on the humoral response of the mice. A dose of 10^7 pfu was chosen for subsequent vaccination since a higher dose caused death in mice (unpublished results, T. E. Toth).

Based on the above preliminary results, the highest dose of 10^7 pfu of the recombinant (WRSBGroEL) was used in a controlled immunization experiment; mice from these groups were subjected to bleeding as described in Materials and Methods section. After each bleeding sera from the groups of mice were analyzed by Western Blot analysis. While sera from WRSBGroEL injected mice did not recognize GroEL antigen until the 7th week, they did react with other vaccinia virus proteins (Fig 8 and Fig 9). Sera from WRSC11 injected mice reacted only with vaccinia virus proteins. Sera from strain RB51 injected mice recognized several *Brucella* proteins while sera from saline injected animals did not recognize any proteins. After the seventh week, sera from WRSBGroEL injected mice reacted specifically with GroEL as well as with other vaccinia virus proteins (Fig 9).

An analysis of the humoral response in mice was performed after virulent bacterial challenge with *B. abortus* strain 2308 which occurred at the 8 weeks post-immunization. In order to determine the effect of challenge on the humoral immune response, comparative Western Blots were done (Fig 10 and Fig 11). Pre- or postchallenge sera obtained either from WRSBGroEL or RB51 vaccinated animals were used to probe a Western Blot of proteins from vaccinia virus, WRSBGroEL and WRSC11 (Fig 10). This comparative Western Blot analysis indicated that reaction of prechallenge sera to GroEL protein was stronger than postchallenge sera reaction. In order to confirm the previous Western Blot results, a second Western Blot was prepared with *B. abortus* RB51, WRSBGroEL and WRSC11 proteins. These antigens were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and cut into strips that contained all three antigenic preparations. Each strip was reacted either with pre- or postchallenge sera (Fig 11). The anti-GroEL responses of pre- and postchallenge sera from

WRSBGroEL and strain RB51 injected mice were compared. In the case of strain RB51 vaccinated mice, postchallenge sera reacted with the GroEL in RB51 much more strongly than prechallenge sera as expected. However, with the WRSBGroEL antigenic preparation, the opposite happened i.e., prechallenge sera reacted stronger than post challenge sera. Similarly, when membranes were developed with pre- or postchallenge sera from WRSBGroEL injected mice, the GroEL response to the strain RB51 antigen was stronger when it was developed with postchallenge sera as opposed to prechallenge sera. Moreover, with WRSBGroEL antigens, the GroEL response was much stronger with prechallenge sera than with postchallenge sera. In other words, the response to GroEL in RB51 was increased after challenge while response to GroEL in vaccinia virus antigen was decreased. Since these Western Blots were done with the same antigenic preparations, transferred and developed using the same conditions, the differences in the GroEL recognition is comparable. Because of these confusing results, the humoral response analysis was repeated with a new set of mice. Variable recognition of GroEL antigen with pre- or postchallenge sera obtained from either WRSBGroEL or *B. abortus* strain RB51 inoculated mice, was not observed; this time postchallenge sera either obtained from WRSBGroEL inoculated mice or RB51 inoculated mice recognized GroEL antigen stronger than the prechallenge sera and also reacted with some other lower molecular weight *Brucella* proteins (Fig 12).

Column Purification of GroEL Antigen for Cell Mediated Immune Response Analysis

Subcloning of 1.57 kb *Brucella groEL* gene into pMALC₂ vector is illustrated in Fig 13. This recombinant vector, pMALC₂GroEL, allowed synthesis of GroEL antigen as a MalE::GroEL fusion protein which was subsequently purified with an amylose resin column (see Materials and Methods). The eluted fractions from the amylose column were collected and examined for purity by Western Blot analysis using either sera collected from mice or goat *Brucella* hyperimmune serum. In Fig 14 the membrane was reacted with goat *Brucella* hyperimmune serum (No. 48) and revealed that a MalE::GroEL fusion protein was expressed as a 97 kDa protein. The protein bands in fractions 2 and 3 between 50-97 kDa are presumed to be degraded MalE::GroEL fusion protein. The concentration of purified protein was determined after consecutive filtration steps and found to be 0.603mg/ml and estimated to be 4% of the crude extract.

Protection Analysis of Vaccinates Against Virulent *B. abortus* 2308 Strain Challenge:

The clearance of *B. abortus* 2308 from mice was determined by assessing viable *Brucella* recovered from spleens and grown on Trypticase Soy Agar™ plates. The cfus of each dilution for each mouse spleen was determined and averaged. These values were graphed to compare the efficacy of the vaccinia virus recombinant as a vaccine candidate relative to the vaccine *B. abortus* strain RB51 and the negative control saline group. The bar graph (Fig 15) shows the average log values of cfus of each group of mice spleens. These log values were analyzed in student paired t-

test (Sigma Plot™) and indicated that the difference in cfus between strain RB51 and saline group treatments was the only statistically significant change (P<0.01) i.e., protection. In contrast, there were no significant differences between the cfus from WRSBGroEL and WRSC11 vaccinated animals relative to the saline treatment.

Lymphocyte Transformation Assays

The spleen from one mouse from each group (Materials and Methods, Immunization Section) was used in the lymphocyte transformation assays. The proliferation of spleen lymphocytes were determined as stimulation indices (SI) calculated as:

$$\frac{\text{insoluble}[H^3] \text{ thymidine cpm of spleen cells exposed to antigen}}{\text{insoluble } [H^3] \text{ thymidine cpm of spleen cells unexposed to antigen}}$$

Antigen Stimulation Indices

Vaccine	ConA ^a	RB51 ^b	GroEL ^c
RB51	16.93	12.53	2.66
WRSBGroEL	5.83	<1	<1
WRC11	16.2	2.4	1.44
Saline	38.9	5.51	5.17

concentrations used: a=1µg/ml, b=10µg/ml, c=10µg/ml

Based on these preliminary LTA results (above) RB51 antigen was able to induce proliferation of lymphocytes of mice inoculated with *B. abortus* strain RB51 (SI=12.53). In contrast, GroEL antigen was unable to induce any proliferation of lymphocytes of either RB51 or WRSBGroEL vaccinated mice. The SI values for Con A (positive control) indicates the lymphocytes from various spleens were capable of proliferation.

Chapter 4

Discussion:

In this work the *B. abortus groEL* gene was successfully cloned into the vaccinia virus WR strain genome. The *groEL* gene was recombined into the vaccinia virus genome by homologous recombination. Based on the results of several Western Blots developed with a known polyclonal *Brucella* hyperimmune goat antiserum (No.48), GroEL expression in the vaccinia virus recombinant produced a 60 kDa protein. Different doses of recombinant virus WRSBGroEL (10^4 , 10^6 , 10^7 pfu per mouse) were tested for infectivity and ability to induce pox lesions on mice skin. All the mice survived without any lesions.

The vaccinia virus/*B. abortus groEL* recombinant, WRSBGroEL, was used to immunize mice to assess the type of immunity induced. Several weeks after the inoculation of the vaccines, sera obtained from these mice were analyzed for their ability to recognize GroEL. Sera failed to recognize GroEL antigen for the first seven weeks after the immunization (whereas other vaccinia virus antigens were recognized) but reacted strongly to GroEL following the seventh week. The reason for the delay of humoral response can not be readily explained by either the dose or the immunogenic efficiency of the recombinant. The lack of a humoral response during the first seven weeks could be correlated with the development of the mice immune system. However this is not likely as, the mice were inoculated at the fourth week of age and considered to have a competent immune system. As the GroEL protein is highly conserved in all eukaryotic and prokaryotic cells, (18, 20, 24) the similarity may be the reason why *Brucella* GroEL protein was not recognized as foreign until after than seven weeks post-immunization. In support of this argument, a previous experiment with six >8 months old mice vaccinated with WRSBGroEL revealed that serum obtained after the third week of immunization recognized GroEL antigen.

Mice were challenged after nine weeks with *B. abortus* 2308 and the efficacy of WRSBGroEL recombinant inducing protective immune response was analyzed by measuring the clearance of *Brucella* from mice spleens. Only the clearance of virulent *B. abortus* from strain RB51 inoculated mice was significantly different from the negative control group and indicated protection. The vaccinia virus recombinant receiving group of WRSBGroEL, administered either IP or ID, did not induce significantly different clearance when compared to the negative control saline group. The samples passed the normality test and the power of the statistical test was acceptable (less than 0.01). Therefore, the vaccinia virus/*B. abortus* GroEL recombinant WRSBGroEL did not protect mice from *Brucella* infection. Although the results of the statistical test indicated that positive protection control group, *B. abortus* strain RB51 inoculated mice, were significantly different from the saline group, the difference in cfu among these two groups was only a half log (saline: 5, RB51: 4.5 \log_{10} cfu/spleen). This difference is very low compared to previous studies with vaccine strain RB51 in which vaccinated mice exhibit differences of 1.5-2 logs compared to with saline immunized mice(3,6,35). The lack of protection by WRSBGroEL could be due to two possibilities: a) vaccinia virus as a live vector is not appropriate for *B. abortus* GroEL antigen presentation or b) *B. abortus* GroEL antigen is not protective against Brucellosis. Although GroEL antigen has been shown to be a protective immunogen with other bacteria (29,39,42) it may not be sufficient to induce protection against a *Brucella* infection. These studies have shown that the recombinant virus is replicating efficiently in mice and can induce the production of GroEL antibodies. This immune response was confirmed using several different antigens (RB51, WRSBGroEL, MalE::GroEL fusion protein) and different antisera (mice, hyperimmune goat).

The differences of the humoral response of pre- or postchallenge sera from either WRSBGroEL or RB51 inoculated mice was assessed. Although reaction to GroEL appeared stronger or weaker in some Western Blots, immunoblots are not quantitative but rather qualitative assays. Therefore, variable results obtained with sera from immunized animals does not reflect the appropriateness of vaccinia virus for antigen presentation. The quantitative differences in the humoral response due to challenge could be analyzed by Enzyme Linked Immunosorbent Assays (ELISA) to more accurately reveal the extent of the immune response.

In order to answer the question as to why this GroEL recombinant is not protective, the cell mediated immune response of the mice must be assessed. In this study preliminary attempts to do such an assessment were carried out. Two different assays were used to assess the cell mediated immune response. A delayed type hypersensitivity (DTH) (skin) reaction was performed (on three mice from each group at the third week following vaccination) by injecting the MalE::GroEL fusion antigen injection into their footpads. No swelling reaction was observed after 48 hours. This experiment was performed without a positive control that would induce a positive DTH reaction. Therefore, we can not be sure if the lack of response is because of the antigen did not induce any reaction in T-lymphocytes of the sensitized animals or animals did not respond at all. Another reason for the lack of response could be because of the prepared antigen (MalE::GroEL) was missing 43 terminal amino acids of GroEL. In lymphocyte transformation assay, the results showed that whole cell RB51 antigen was able to induce proliferation of spleen cells harvested from mice vaccinated with *B. abortus* strain RB51. This showed that RB51 antigen was a good immunogen that stimulated lymphocyte proliferation; however, the reaction was not specific as saline vaccinated mice showed an increased SI. Therefore, the RB51 antigen dose should be established in order to see if there is a specific response. In contrast purified GroEL antigen did not induce proliferation of the lymphocytes from mice vaccinated with any preparations. Again saline inoculated mice exhibited a low stimulation index suggesting a non-specific reaction. The inability of the GroEL to stimulate lymphocyte proliferation should not be conclusively ruled out until purified GroEL (including the entire protein and not fused to MalE) is tested over a concentration range.

In conclusion, the *B. abortus groEL* gene was successfully cloned into and expressed by vaccinia virus. Recombinant WRSBGroEL replicated efficiently in mice and induced a specific humoral response to *Brucella* GroEL and vaccinia virus antigens. However, the recombinant vaccinia virus did not protect mice from virulent *B. abortus* 2308 challenge under the conditions tested.

Chapter 5

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Chapter 6

Figures

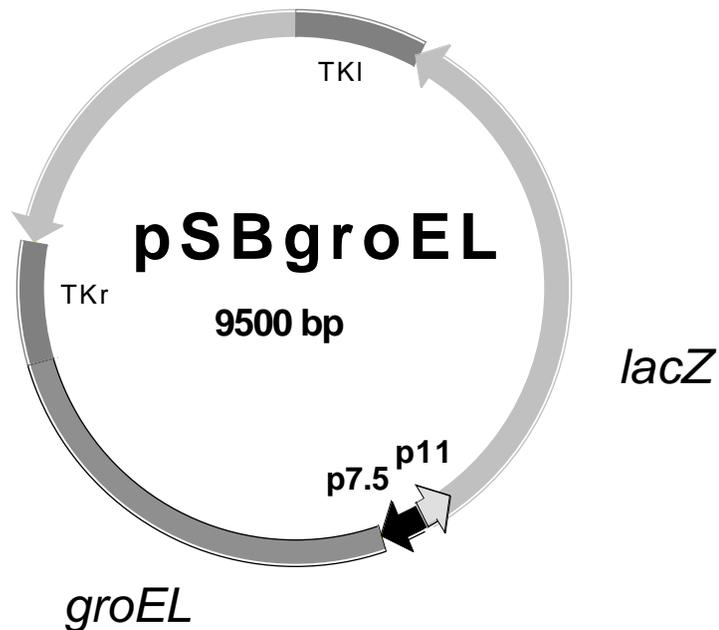
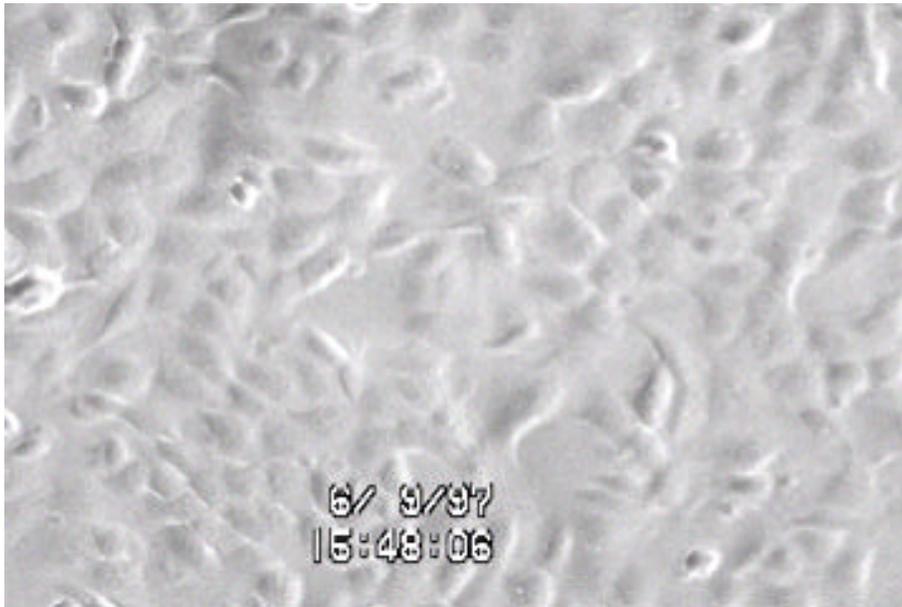


Fig 1. Diagram of recombinant plasmid pSBGroEL. A 1.7 kb fragment containing the *B. abortus* *groEL* gene (cross hatched line) was cloned into the shuttle vector pSC11 (light gray regions). The early/late vaccinia virus promoter p7.5 (solid arrow) regulates expression of the *groEL* gene and a late vaccinia virus promoter p11 (dotted arrow) regulates the expression of the *lacZ* gene. The vaccinia virus thymidine kinase sequences (TKI, TKr) flank the expression cassette controlled by the p7.5 and p11 promoters.

A



B

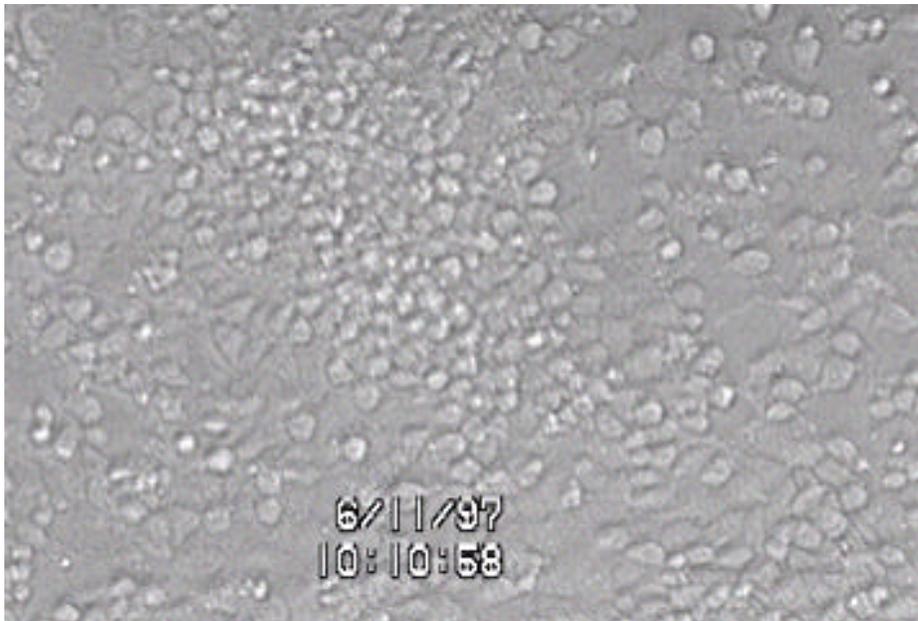


Fig 2. Panel A shows a regular HuTK⁻ cell culture at about 90% confluency. Panel B shows the same cells after 42 hours of infection with recombinant WRSBGroEL (MOI 0.2).

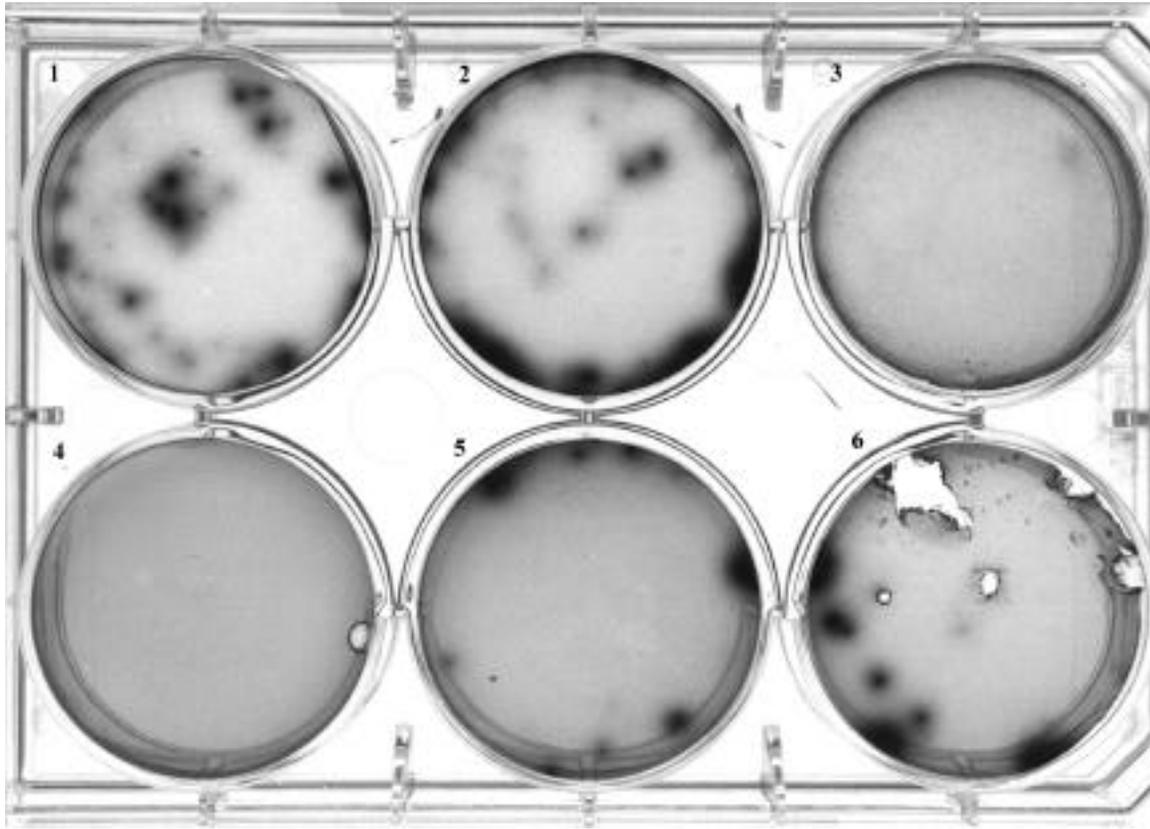


Fig 3. Plaque purification of vaccinia virus/*B. abortus groEL* recombinant. First two wells are the 10^{-3} dilution and the third well is the 10^{-4} dilution of the recombinant virus preparation at the tertiary plaquing step. Fourth well is the cell control for the plaque assay; the fifth and sixth wells are 10^{-3} dilutions of another stock of recombinant WRSBGroEL. Darker spots (representing beta-galactosidase expression) in the wells are recombinant virus plaques. Well 6 shows plaques removed for subsequent plaque purification steps.

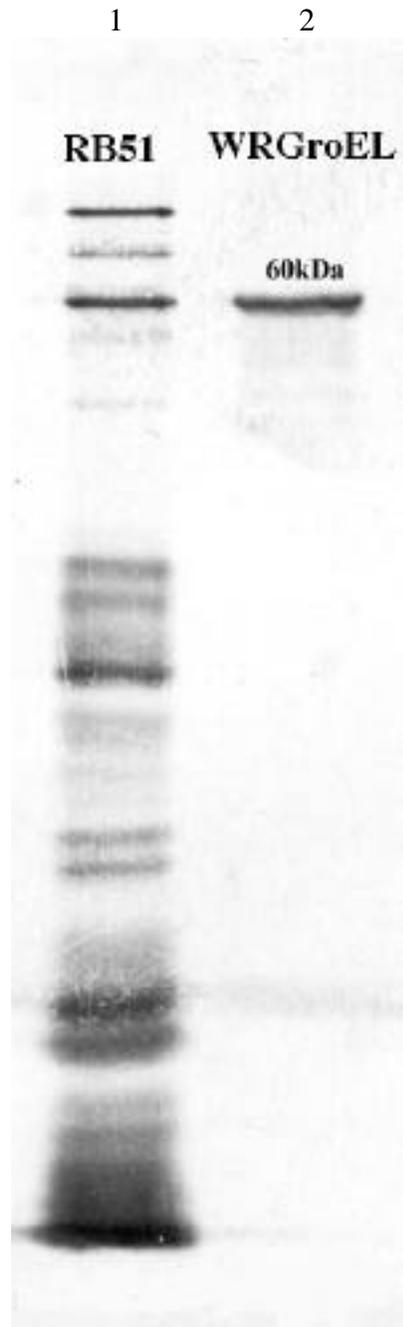


Fig 4. Western Blot analysis of the vaccinia virus/*B. abortus* groEl recombinant. *Brucella* hyperimmune goat anti-serum (No.48) was used to detect specific GroEL protein (60kDa). Lane 1 contains proteins from *B. abortus* RB51; Lane 2 contains proteins from HuTK⁻ cells infected with recombinant vaccinia virus WRSBGroEL.

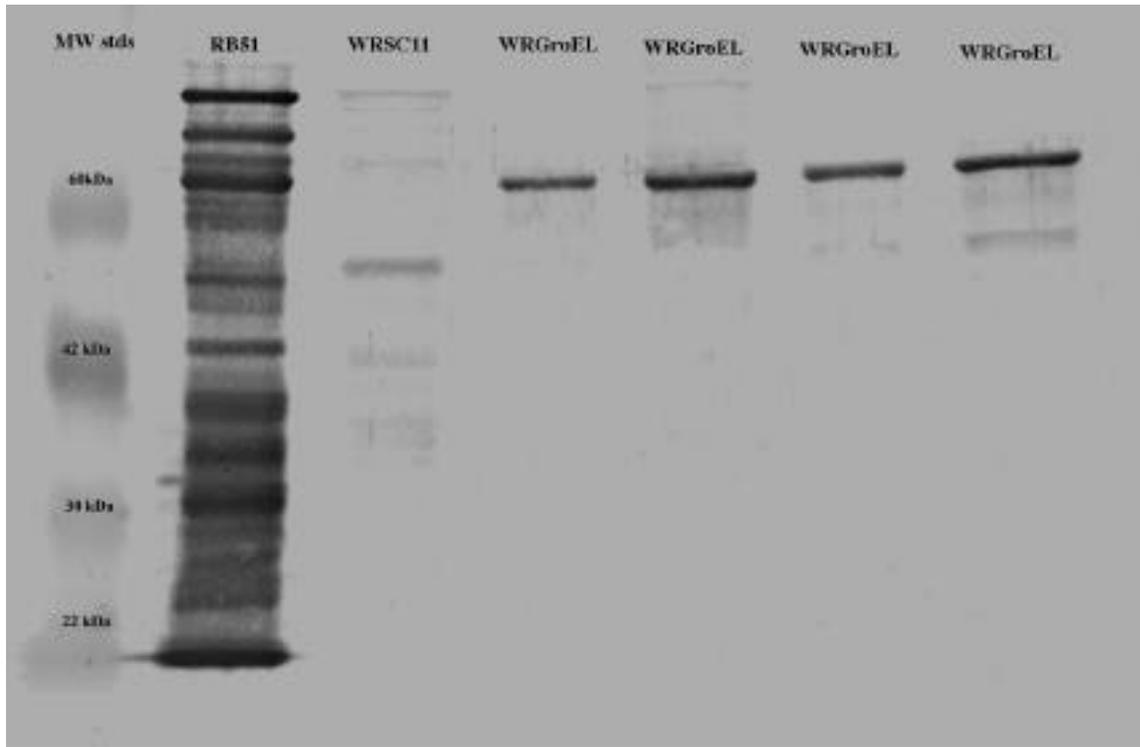


Fig 5. Western Blot analysis of vaccinia virus recombinant WRGroEL (four different stocks) and plasmid control (WRSC11) and *B. abortus* RB51 antigens. *Brucella* hyperimmune goat serum No.48 was used to detect the proteins.

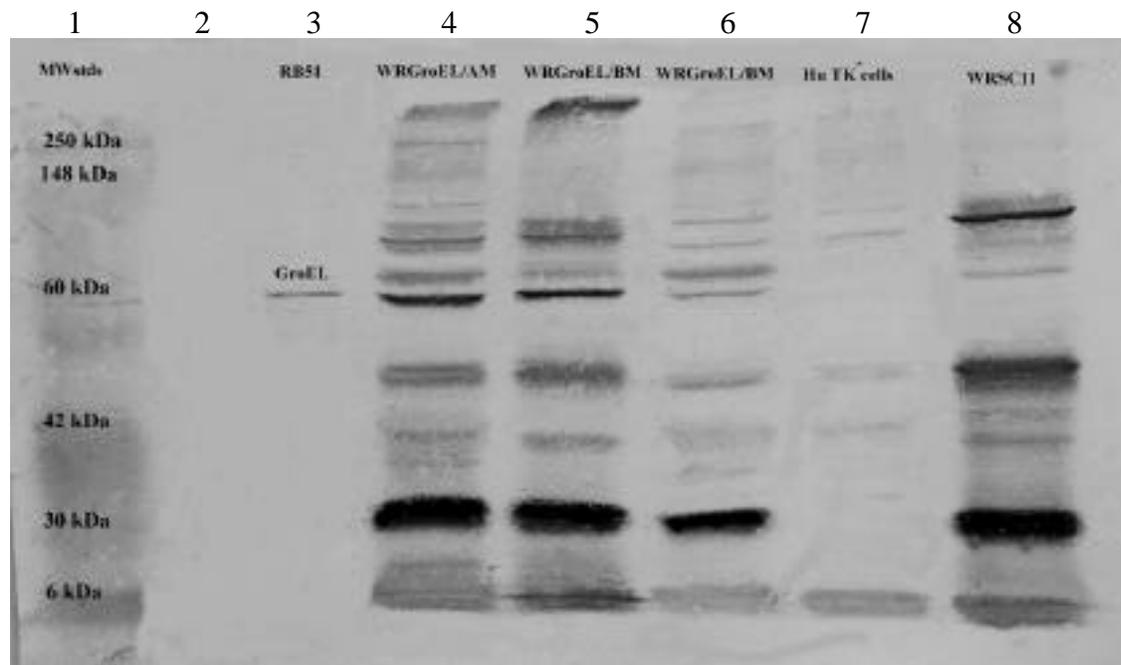


Fig 6. Western Blot analysis of vaccinia virus and *B. abortus* proteins using sera from recombinant WRSBGroEL (10^4 pfu) inoculated mice. Samples contain proteins from *B. abortus* RB51(lane 3); from vaccinia virus/*B. abortus groEL* recombinant after purification from mice(AM) (lane 4); recombinant proteins before purification from mice (BM) (lanes 5, 6); human thymidine kinase negative cells (HuTK⁻) (lane 7) and proteins of HuTK⁻ infected with plasmid control vaccinia virus recombinant WRSC11 (lane 8).

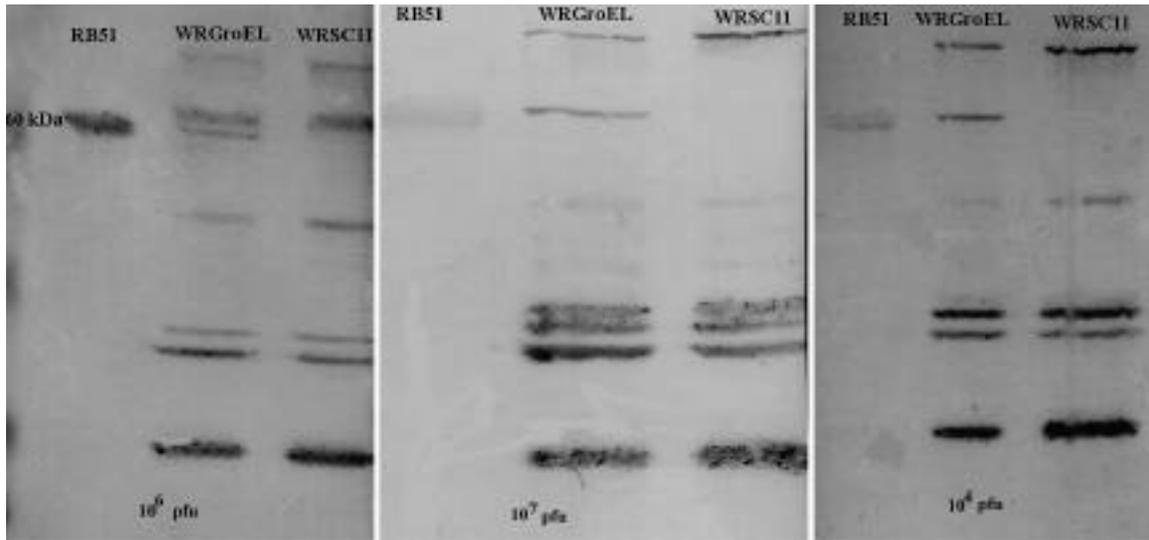


Fig 7. Western Blot analysis of vaccinia virus and *B. abortus* strain RB51 proteins using sera from WRSGroEL vaccinated mice. Mice were inoculated with different recombinant vaccinia virus doses (10^4 , 10^6 , 10^7 pfus). Three individual Western Blots are placed side by side in order to compare the humoral responses of the mice to the recombinant virus dose; antigens were *B. abortus* strain RB51 (RB51), vaccinia virus WRSGroEL (WRGroEL) and vaccinia virus WRSC11 (WRSC11); the dose (pfu) is indicated at the bottom of the blots.

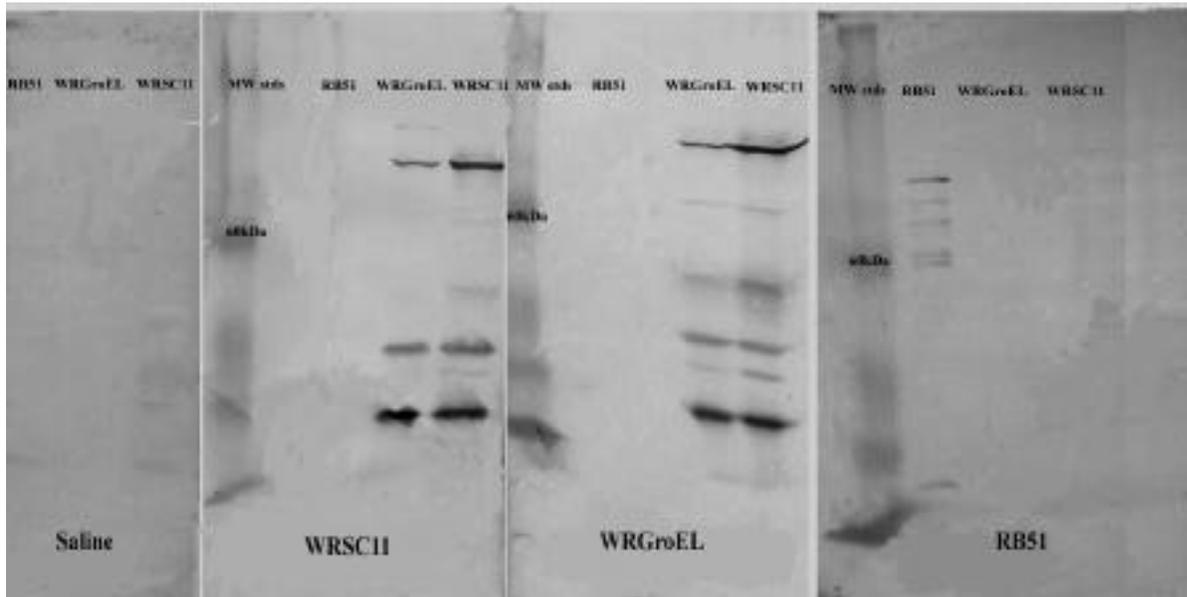


Fig 8. Western Blot analysis of WRSBGroEL, WRSC11 and *B. abortus* strain RB51 proteins. Blots are probed with sera following the fifth week post vaccination; the type of vaccine injected is indicated at the bottom of the membrane. The types of antigen preparations are *B. abortus* strain RB51 (RB51), vaccinia virus WRSBGroEL (WRGroEL) and vaccinia virus WRSC11 (WRSC11) and molecular weight standards (MW stds).

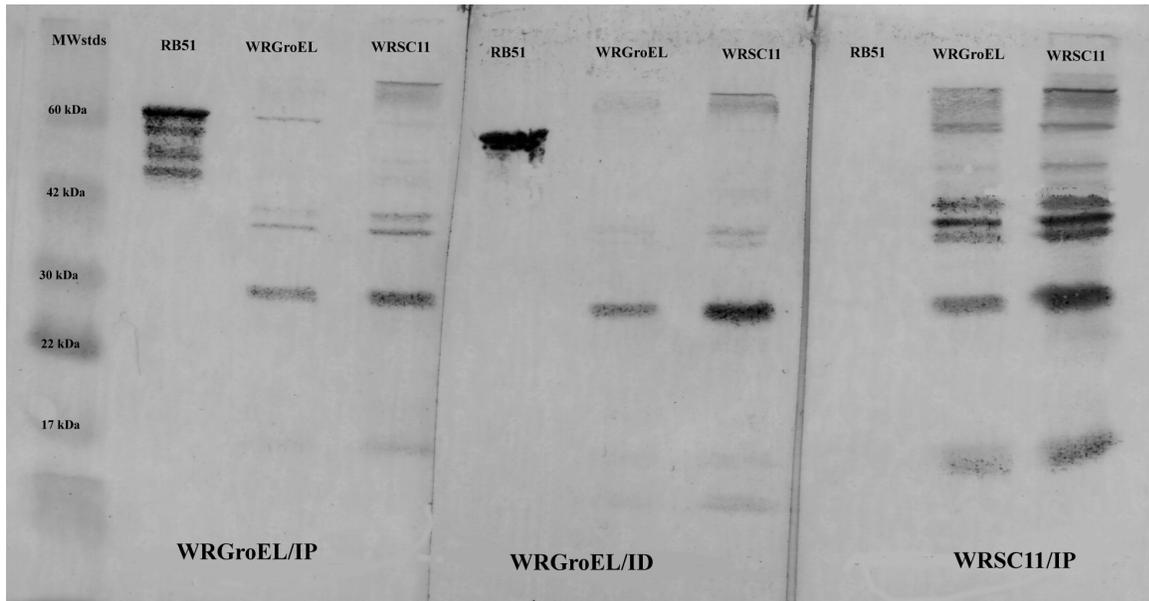


Fig 9. Western Blot analysis of mice sera at the seventh week following vaccination. Blots were probed with sera from mice vaccinated intraperitoneally (IP) with WRSBGroEL, intradermally (ID) with WRSBGroEL or IP with WRSC11, respectively. The preparations are *B. abortus* strain RB51 (RB51), WRSBGroEL (WRGroEL), WRSC11 (WRSC11) recombinants and molecular weight standards (MWstds).

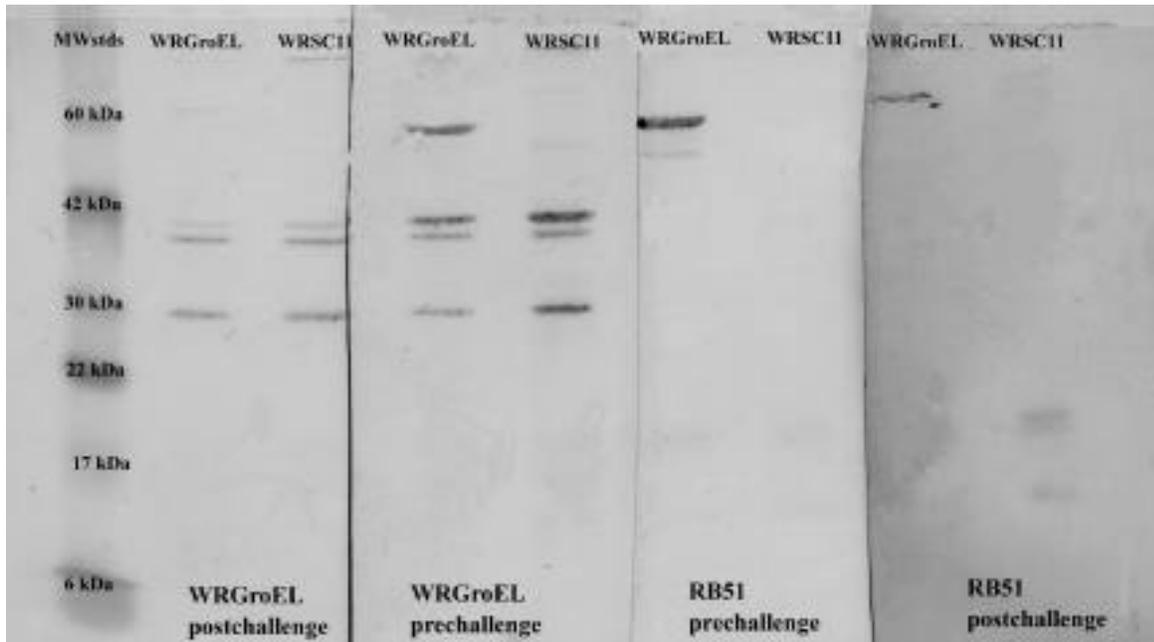


Fig 10. Western Blot analysis of vaccinated mice challenged with virulent *B. abortus* strain 2308. Sera were obtained prior to and after the challenge from either WRSBGroEL or RB51 vaccinated mice; the type of vaccine injected and the time in relation to challenge is indicated at the bottom of the membranes. Antigen preparations are WRSBGroEL (WRGroEL) and WRSC11 (WRSC11) recombinants.

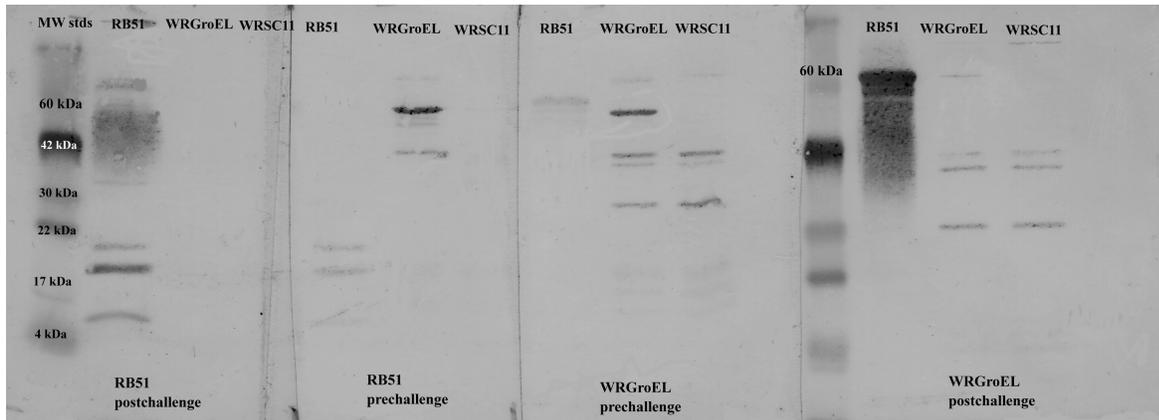


Fig 11. Western Blot analysis of vaccinated mice challenged with virulent *B. abortus* strain 2308. Sera were obtained from prior and after the challenge from either WRSBGroEL or RB51 vaccinated mice; the type of vaccine injected and the time in relation to challenge is indicated on the bottom of the membranes. The types of antigen preparations are WRSBGroEL (WRGroEL) and WRSC11 (WRSC11) recombinants, *B. abortus* strain RB51 (RB51) and molecular weight standards (MW stds).

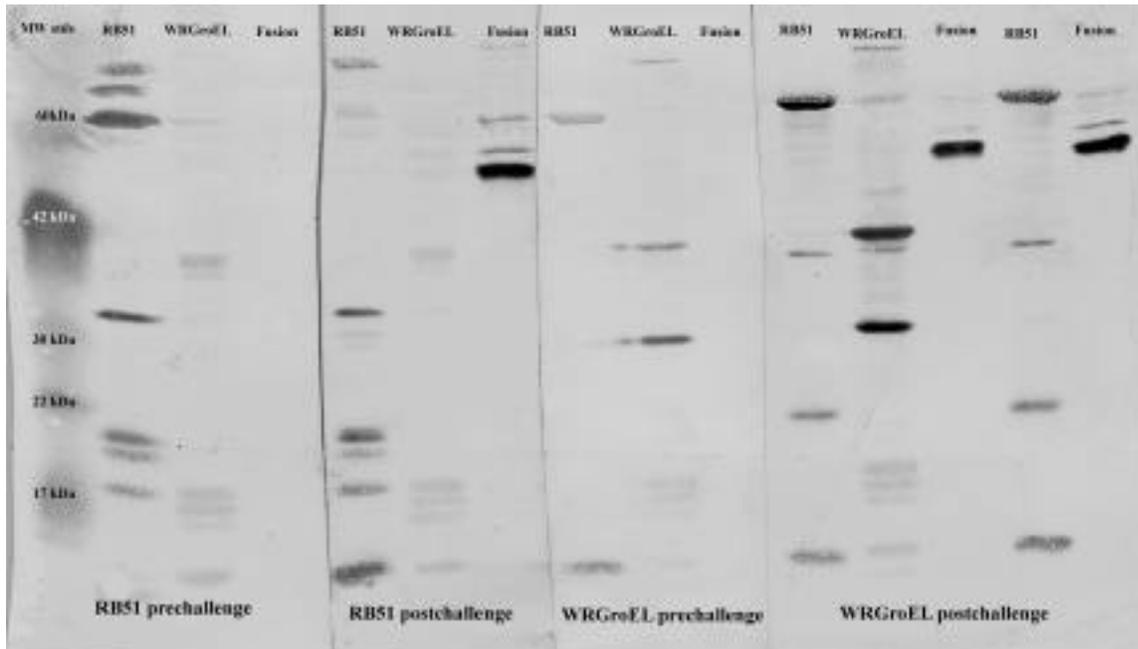


Fig 12. Western Blot analysis of vaccinated mice challenged with virulent *B. abortus* strain 2308. Sera were obtained prior and after challenge and the time in relation to challenge from either WRSBGroEL or RB51 vaccinated mice, the type of vaccine injected is indicated on the bottom of the membranes. Antigen preparations are WRSBGroEL (WRGroEL), *B. abortus* strain RB51 (RB51), GroEL::MalE fusion protein (fusion) and molecular weight standards (MW stds).

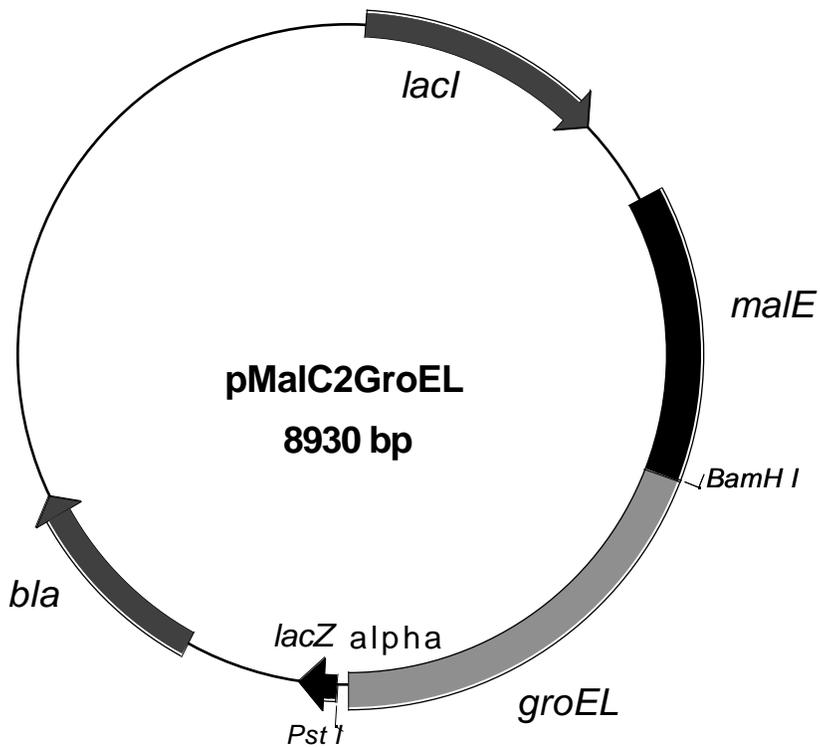


Fig 13. Diagram of recombinant expression plasmid pMalC2GroEL. A 1.57 kb fragment containing the *B. abortus groEL* gene (cross hatched line) was amplified by polymerase chain reaction (PCR) and subcloned into the *BamH* I, *Pst* I sites of pMalC2, fused to *malE* gene just upstream of *lacZ* alpha peptide gene (solid black arrow); the *bla* gene encodes beta lactamase, *lac* I gene encodes for Lac operon suppressor.

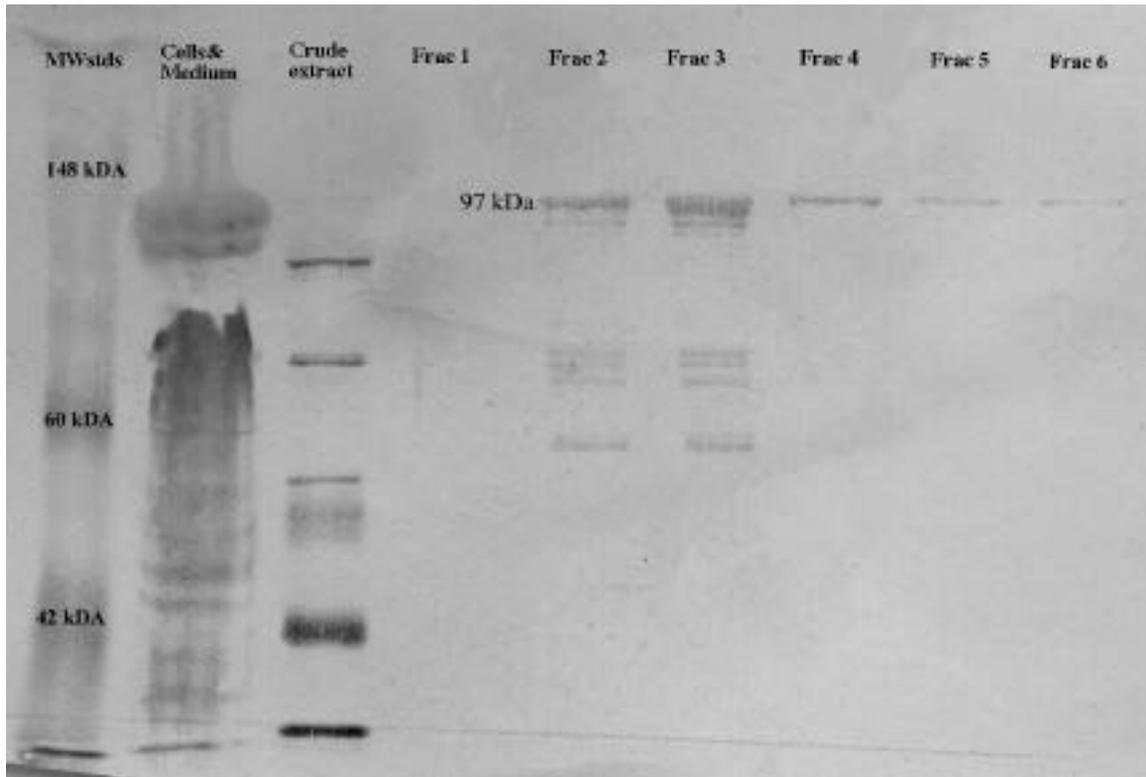


Fig 14. Western Blot analysis of Male::GroEL antigen preparations. Lanes contain molecular weight (MW) standards, cells and medium, crude extract, and eluted fractions (frac)1-6. The blot was reacted with goat *Brucella* hyperimmune serum (No.48).

CFUs/spleen

log10

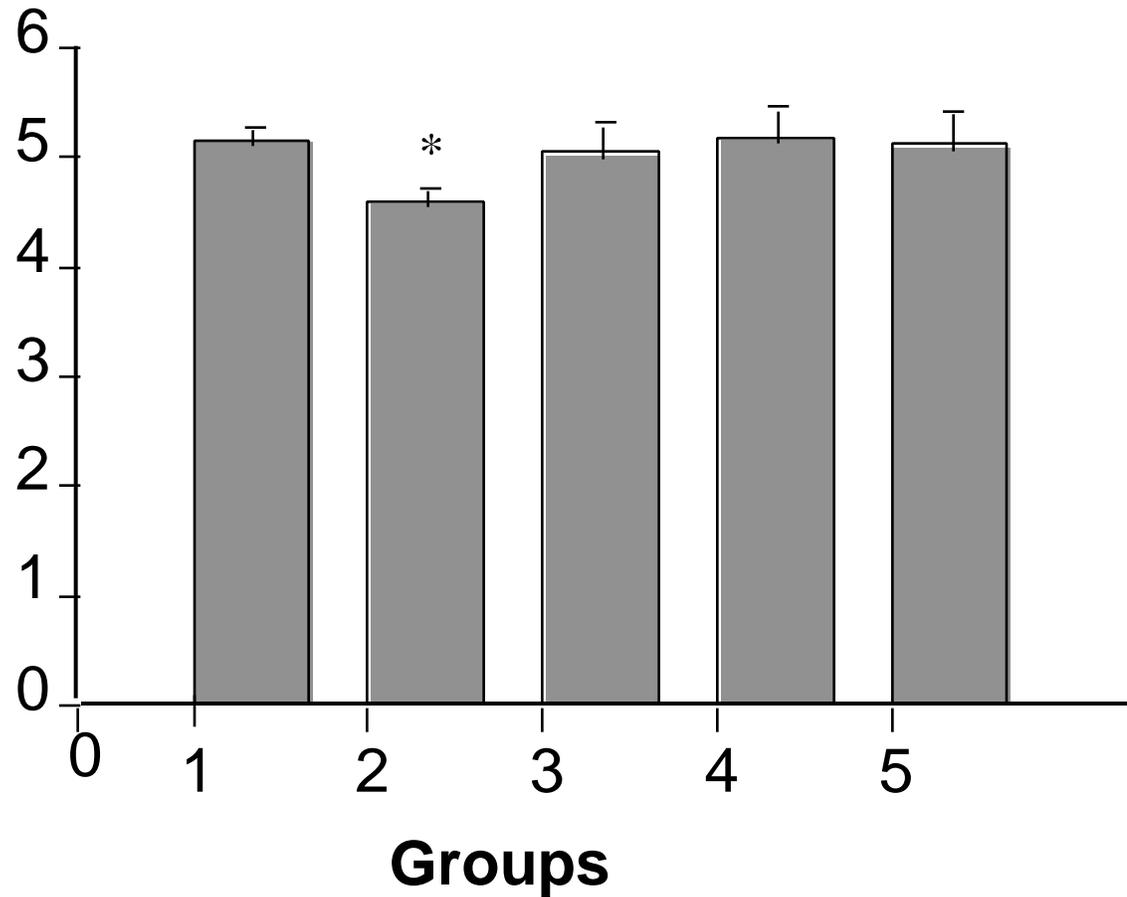


Fig 15. Bar graph of *B. abortus* 2308 cfus in mouse spleens 2 weeks following challenge. Groups of mice were vaccinated with (1) vaccinia virus WRSBGroEL intradermally; (2) *B. abortus* RB51 intraperitoneally; (3) saline intraperitoneally; (4) vaccinia virus WRSBGroEL intraperitoneally and (5) vaccinia virus WRSC11 intradermally. Doses of either vaccinia virus or *Brucella* used are described in Materials and Methods section. The bars on each group indicates the standard deviation. * P<0.01

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ABSTRACTS and PRESENTATIONS:

S. Ozalp, J. R. McQuiston, N. Sriranganathan, G. G. Schurig, S. M.
Boyle. Genetic analysis of the *lys A* gene of *Brucella abortus*. Seventh
Annual Research Symposium May, 1995. VMRCVM, Blacksburg, VA.

S. Baloglu, J. R. McQuiston, N. Sriranganathan, G. G. Schurig, S. M.
Boyle. Genetic analysis of the *lys A* gene of *Brucella abortus*. A.S.M.
Virginia Branch December, 1995. Charlottesville, VA.

S. Ozalp, J. R. McQuiston, N. Sriranganathan, G. G. Schurig, S. M.
Boyle. Genetic analysis of the *lys A* gene of *Brucella abortus*. Poster
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Genomic library expression, screening.
Restriction mapping of plasmids.
Horizontal Agarose Gel Electrophoresis.
Protein isolation and purification
SDS Poly-Acrylamide Gel electrophoresis.
Southern Blotting and probing of DNA gels.
Western Blotting.
Immunoblotting using mono and polyclonal antibodies.
Cloning in Expression vectors.
Growth and maintenance of eukaryotic cell cultures.
Transfections, plaquing and enhancements of viral recombinants.
Preparing virus stocks and M.O.I. determination.
Antibiotic and color selection.
Immunizations and immune assays using mouse model.
Lymphocyte Transformation Assay
Use of DNA analysis software.
Certified for Biosafety Level 3 procedures and Radioactivity.

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