Development of an Antibiotic Resistance Free Bivalent Vaccine Against Swine Brucellosis and Swine Influenza

Parthiban Rajasekaran

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Dr. Stephen M. Boyle, Chair
Dr. Nammalwar Sriranganathan
Dr. Gerhardt G. Schurig
Dr. Biswarup Mukhopadhyay
Dr. Paul C. Roberts

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ABSTRACT

Livestock across the world contract several infectious diseases of both bacterial and viral origin. Swine brucellosis caused by *Brucella suis* and swine influenza caused by Influenza A virus affect both domestic and feral swine populations. Both the diseases have zoonotic potential to cause disease in humans with serious complications apart from inflicting huge economic losses. Infected feral swine can also act as a source of spread and outbreak where the disease is not endemic. At present, there is no vaccine available for swine brucellosis. The currently used swine influenza vaccine may not be effective against influenza strains like the recent H1N1 strain that caused a pandemic. To develop an effective bivalent vaccine for swine against these two diseases, a leucine auxotroph of the USDA approved vaccine *B. abortus* strain RB51 was constructed along with *leuB* gene complementing plasmid pNS4 to over-express antigens from *Brucella* and influenza. This antibiotic resistance free system over-expressed *Brucella* derived antigens SOD, L7/L12 and WboA in three different constructs. Against a virulent challenge of *B. suis,* the candidate vaccine strain over-expressing both SOD and WboA protected mice more significantly than the control group and was also found to be better protective than other candidate vaccine strains over-expressing either SOD and L7/L12 together or SOD alone. Immunoassays (ELISA) suggested that the protection afforded is Th1 type mediated immune response, as cytokine IFN-γ and IgG2a antibody sub-isotype was observed in the splenocyte culture supernatant and serum samples respectively. The strain

RB51*leuB* platform was not expressing influenza derived antigens Hemagglutinin (HA) and Nucleoprotein (NP) when screened for expression by immunoblot. Influenza antigens, HA, NP and ectodomain of matrix protein M2e, were not found to be expressing even after optimizing their codon usage to suit *Brucella* tRNA preference. However, RT-PCR showed that the influenza genes mRNA were produced. In conclusion, this dissertation describes the construction of an environmentally safe antigen over-expression platform and successful employment of the system as a candidate vaccine in protecting mice against *B. suis* challenge. This new platform is a potential candidate for developing vaccines against other infectious diseases of livestock. This document also discusses alternate strategies for expressing influenza antigens in a *Brucella* platform.

Dedication

To **“Mother Nature”**

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Chapter 1

Introduction and Rationale

"Prevention is better than cure" – As the idiom goes, vaccines have proved to be the most effective tool in the containment and eradication of once devastating diseases like small pox, poliomyelitis, pertussis in humans and rinderpest (cattle plague) in animals. Vaccinating animals against pathogens, particularly zoonotic agents, not only helps the farmer to avoid financial losses but also prevents spread of the disease to humans (35). Multivalent vaccines have been used both in human and veterinary medicine as a cost saving measure and as well as an effective disease prevention strategy by protecting against multiple infectious agents (29). The currently used childhood human MMR vaccine (against measles, mumps and rubella) and the canine DHLPPi vaccine against canine distemper, infectious canine hepatitis, leptospirosis, parvovirus and parainfluenza, are some examples of successful multivalent vaccines. Multivalent vaccines are prepared either as a cocktail of two or more live attenuated viruses (ex. MMR), killed pathogens and/or toxoids of pathogens (ex. DTP used against diphtheria, tetanus and pertussis). Multivalent or polyvalent vaccines are also developed by over-expressing homologous or heterologous antigenic determinants on a vaccine vector (bacteria or virus) to elicit protective immune response against multiple pathogens (1, 14).

Live attenuated bacteria (Gram-negative and Gram-positive) are used as vectors to deliver homologous (antigens from the same genus) or heterologous (genes from other

bacteria or virus or parasite) antigens thus making other types of polyvalent vaccines (6). Bacterial vectors used to deliver antigens should posses the desirable characteristics of an effective vaccine that includes: the ability to induce long-term protective immunity, poses no danger or adverse reactions to the recipient, be in-expensive and easily produced and stored, and exhibit minimal or no interference with diagnostic tests (4, 30). Using bacteria as vaccine vectors has the advantage of delivering the antigen inside the host at the specific site or tissue involved (9). Additionally, the bacterial vector's antigens act as natural adjuvant to the delivered foreign antigen (16). Live attenuated strains of *Salmonella* spp., *Mycobacterium bovis* BCG, *Listeria monocytogenes*, *Shigella spp.* and *Vibrio cholerae* have been shown to deliver heterologous antigens from other pathogenic bacteria, viruses and parasites (28). Some of the above-mentioned bacteria, like *Salmonella* and *Mycobacterium* have an intracellular infection stage in their life cycle, which allows an attenuated form of the same bacteria to deliver antigens in an intracellular location (30). An advantage of using intracellular bacteria like *M. bovis* BCG, as an antigen-delivering platform, is that they can elicit B-cell based antibody response during the extracellular stage of infection and once they are intracellular, can elicit a T-cell immune response (9). Owing to this advantage, *M. bovis* BCG has been investigated to deliver HIV antigens in the process of developing a vaccine against AIDS (3). Heterologous antigens secreted into the host system or presented on the surface of the delivering intracellular bacteria elicit a better and quicker immune response than the antigen that is presented within the cytoplasm. The cytoplasmic antigen will only be encountered by the host immune system after the bacterial lyses (4, 9, 13). Fusion of foreign antigens to signal sequence of proteins that will be transported to cell wall of the

bacterial vector will help present antigen to the immune system continuously until the bacteria is killed and thereby resulting in a prolonged antigen stimulation period (9). One of the effective strategies is to use the secretion apparatuses of bacteria like type III secretion system of *Salmonella* and *Yersinia* to deliver antigens into the host cell cytoplasm (19).

Medium copy number or high copy number plasmids are used to over-express antigens in a bacterial platform without being toxic to the carrier strain (30). In some instances, to overcome the issues related to plasmid stability inside bacteria after vaccination, genes encoding heterologous antigens are integrated to the chromosome of the bacterial vector (8). Since *Mycobacterium* possesses the qualities of a vaccine vector it has been investigated to express foreign antigens that are integrated into its genome (8). However, antigens that are over-expressed on a multi-copy number plasmid inside a bacterial vector are more likely to be antigenic and elicit a stronger immune response than a single copy of antigen cloned on to the chromosome (2, 30). The single copy of the heterologous antigen on the chromosome may not be sufficiently antigenic to elicit a potent innate immune response as well as a long lasting memory cell mediated immune response (30).

Attenuated strains of *Brucella spp*. possess the characteristics of an antigen delivery vehicle that could elicit a protective immune response against a plasmid borne over-expressed antigen. A live attenuated strain of the Gram-negative, facultative intracellular bacterium *B. abortus*, strain RB51, is the USDA approved vaccine against cattle brucellosis (22, 23). A rough derivative of the smooth wild type strain *B. abortus*, strain RB51 lacks the ability to produce the O-side chain associated with the

lipopolysaccharide region of the cell envelope (22). Using rough strain RB51 for vaccination does not interfere with diagnostic test to detect infected animals, as the serological test screens for the presence of antibodies against the cell surface O - side chain antigen (21). Strain RB51 gets phagocytosed and delivers antigens at intracellular level during that process, and thereby can be used as a vaccine vector for delivering antigens from pathogens that need cell mediated immunity (CMI) for protection (17, 21, 31, 32, 34). Strain RB51 has been shown to deliver antigens from *Neosporum caninum* (parasite) and *Bacillus anthracis* apart from over-expressing and delivering *Brucella* derived antigens (17, 18, 31-34). A medium copy number plasmid system (pNS family) that is compatible with *Brucella spp*. for over-expressing cloned foreign antigen was developed as a part of the multivalent vaccine development system using *Brucella* as a platform (27). The pNS group of plasmids expressed a varied range of antigens from multiple pathogens on different bacterial platforms without developing any toxicity issues (24-27). The gene encoding origin of replication employed in this pNS plasmid system is from the pBB plasmid that is compatible with most Gram-negative bacteria and some Gram-positive bacteria thus making this pNS group of plasmids compatible with different bacterial platforms (27). This group of plasmids uses antibiotic resistance cassettes that allow for selection of recombinants on chloramphenicol, kanamycin and ampicillin plates (25-27). Though using antibiotic resistance markers containing pNS plasmid on a *Brucella* platform to over-express antigens are convenient for laboratory manipulations, releasing a vaccine vector containing antibiotic resistance gene into field conditions is strictly regulated (7, 30).

Deliberate release of antibiotic resistance genes into the ecosystem is found to potentiate the horizontal transfer of resistance among microbes (7). Moreover, using antibiotic resistance genes as markers allows for selection of recombinants only under *in vitro* laboratory conditions. But, the absence of selective pressure for plasmid retention after the bacteria is introduced into the host may be metabolically expensive and would eventually end up in plasmid loss due to segregation (4, 7, 30). Thus alternative strategies must be employed to develop a plasmid system that possesses the characteristics: "non-conjugative, preferably non-mobilizable, and harbors no antibiotic resistance marker" (4, 7, 30).

Auxotrophic mutants that lack the ability to synthesize specific genes needed for cell integrity e.g. cell wall could be complemented with a plasmid borne intact gene (acting as marker). *Salmonella* mutants lacking the ability to synthesize diaminopimelic acid (DAP) created by deleting the chromosomal copy of the *asd* gene and complemented with an Asd^+ plasmid were shown to act as an environmentally safe vector-plasmid system for heterologous antigen expression (5). DAP is an integral component of peptidoglycan of the cell wall of bacteria and is not available inside the host cells and so a mutant lacking the ability to biosynthesize DAP cannot survive inside the host cell environment. Similarly, a bacterium's *thyA* gene encoding for thymidylate synthase (necessary for *de novo* synthesis of the DNA precursor thymidine phosphate), when mutated lacks the ability to synthesize DNA on its own. This thymidine mutant can be complemented by a plasmid borne *thyA* gene (15) acting as a marker is another safe platform for antigen expression. Another balanced lethal plasmid system based on a *purB* mutation in *Salmonella spp.* has shown to over-express a *purB* complementing plasmid

and an urease gene from *Helicobacter pylori* (10). The *purB* mutant would not be able to biosynthesize purine by itself (10). An alternative strategy for plasmid retention and non-antibiotic resistance markers includes the *hok-sok* system that is based on toxin-antitoxin module. In this case the bacteria gets killed post-segregation of plasmids thus, providing a selection pressure to retain the plasmid, since the marker gene on the plasmid down regulates a toxic gene on the bacterial vector (30).

Plasmid stabilization systems were also developed based on auxotrophic mutants that lack the ability to biosynthesize an amino acid. Intracellular bacteria that need to biosynthesize all its nutrients may not be able to survive inside phagosomes of the phagocytic cells if one of their genes for amino acid biosynthesis is mutated. Under laboratory conditions, a plasmid carrying an intact *glnA* gene complemented *in trans* the *V. cholerae* Peru2Δ*glnA* mutant that lacks the ability to produce glutamine synthetase (20). Likewise, an alanine mutant of *L. monocytogenes* along with the complementing plasmid has been used to over-express HIV antigens in primate models with reasonable success (12). Pathogenic bacteria like *M. tuberculosis* were attenuated when they were genetically engineered to be auxotrophic for essential amino acid like leucine as they would not survive in a nutrient limiting environment like the phagosome of immune cells (11). Unless leucine was supplemented, intracellular growth and the survival of this leucine mutant, *M. tuberculosis* Δ*leuD,* in mice was impaired (11). Protective efficacy of this leucine auxotroph was comparable to the standard tuberculosis vaccine strain against a virulent challenge under laboratory conditions (11). Thus, constructing a leucine auxotroph of an intracellular bacterium, *Brucella*, would be a viable, efficacious and environmentally safe vector system, if used with a *leuB* complementing plasmid to

deliver homologous and heterologous antigens. The following 3 chapters describe: (i) the construction of this delivery system, (ii) assessment of antigen expression capabilities and (iii) evaluation of the level of protection afforded by the *B. abortus* RB51*leuB* vaccine vector against a virulent challenge in a mouse model.

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Chapter 2

Brucella abortus strain RB51 leucine auxotroph as an environmentally safe vaccine for plasmid maintenance and antigen over-expression

Rajasekaran, P., M.N. Seleem, A. Contreras, E. Purwantini, G.G. Schurig, N. Sriranganathan, and S.M. Boyle. 2008. Appl Environ Microbiol **74:**7051-5.

ABSTRACT

The USDA approved cattle vaccine, *Brucella abortus* strain RB51 has been successfully employed as a platform to over-express protective antigens under experimental conditions. Conventional over-expression systems from a live bacterial vector platform use replicating plasmids containing antibiotic resistance genes as markers for laboratory manipulations. Regulatory bodies for recombinant vaccines do not recommend vaccine vectors that use antibiotic resistance marker, as the vaccine could act as a source of spread of resistance to the normal flora and other pathogens in the introduced environment. Hence, to over-express both homologous and heterologous antigens in a safer way in strain RB51, a replicating plasmid containing no antibiotic resistance gene was constructed. An unmarked *leuB* mutant of the strain RB51 was created by partial deletion of the *leuB* gene using *cre-lox* methodology. The mutation of the *leuB* gene in the strain RB51 caused its inability to synthesize leucine. The leucine deficiency of the mutant was complemented by the plasmid pNS4, which has an intact *leuB* gene replacing the antibiotic resistance marker. A heterologous antigen, green fluorescent protein (GFP), was cloned into the pNS4 as a model antigen and expressed in strain RB51*leuB* under conditions *in vitro* and in mice. The *leuB* auxotroph and the

complemented *leuB* auxotroph were both able to protect CD1 mice against a challenge dose of virulent *B. abortus* 2308; in addition, the complemented strain elicited GFP specific antibodies. In principle, the GFP in this novel plasmid (pNS4/GFP) can be replaced with a protective antigen from any other infectious agent that needs strong cell mediated immunity, thus making the leucine auxotroph of vaccine strain RB51 a safer and more effective multivalent vaccine vector that addresses regulatory issues.

1. Introduction:

B. abortus strain RB51 is an USDA approved vaccine used to control bovine brucellosis in the United States and a number of other countries (31). The vaccine strain RB51 is a stable rough form and a derivative of the smooth wild type *B. abortus* strain S2308 (23). Rough strains of *B. abortus* are attenuated when compared to their parent smooth strains (2, 16, 23). Since the rough strain RB51 lacks O-side chain of the lipopolysaccharide, animals vaccinated with this strain do not produce O-antigen specific antibodies, which allows for distinguishing vaccinated animals from infected animals (31). Moreover, this rough strain elicits protective cell mediated immunity upon vaccination (3, 30). As strain RB51 possesses characteristics of an effective vaccine, it has been investigated as a platform to over-express and deliver homologous (*Brucella* derived) and heterologous (non - *Brucella* derived) antigens (21, 31, 32). In order to use strain RB51 as a multivalent vaccine carrier for inducing immune responses against both homologous and heterologous antigens, a plasmid vector containing the gene encoding the foreign antigen along with an antibiotic resistant gene serving as a plasmid marker had been employed (31, 32). This practice has been criticized as it has the potential to

introduce the antibiotic resistance gene into normal flora as well as pathogens in the vaccinated animals (1, 13, 33). Construction of a plasmid expressing the protective antigens that is not dependent on an antibiotic resistance gene for maintenance would be an acceptable alternative, as it would have a minimum of environmental risk.

Macrophages are known to be a nutrient limited environment for bacteria (4, 9). Therefore, it is unlikely an intracellular pathogen such as *Brucella* that is mutated in a gene for biosynthesis of an essential amino acid will be able to survive inside a macrophage (4, 9). In addition, complementation of this auxotroph with a plasmid carrying the wild type gene (encoding the enzyme necessary for the amino acid synthesis) would provide the selection for maintenance of this plasmid inside of *Brucella* in the macrophages. Marker-less auxotrophic mutants may be used in place of genetically engineered conventional bacterial vaccine strains to avoid the introduction of antibiotic resistance genes into an animal population (33).

Since the phagosomes inside the macrophage are nutrient deficient (7), the intracellular bacteria residing in the phagosome have to synthesis their own nutrients. Leucine auxotrophs of intracellular bacteria have shown to be attenuated inside macrophages (7). Like many other bacteria, *Brucella* also uses the isopropylmalate pathway for leucine biosynthesis (9), governed by four genes *leuA*, *leuB*, *leuC* and *leuD* (Fig. 1). Analysis of the genome sequence in National Center for Biotechnology Information website revealed that unlike in *E. coli* where all the four genes are in a operon (9), *leuA* and *leuC* are on chromosome I and *leuB* and *leuD* are on chromosome II of *Brucella* spp. The *leuA* gene encodes isopropylmalate synthase that converts 2-keto-

isovalerate into 2-isopropylmalate (Fig.1). The precursor 2-keto-isovalerate is a byproduct of valine biosynthesis.

The (2S)-2-isopropylmalate gets dehydrated to become (2R, 3S)-3-isopropylmalate by the activity of the enzymes encoded by the *leuC* and *leuD* genes; the former codes for the large subunit and the later for the small subunit of isopropylmalate dehydratase. The 3-isopropylmalate is converted into 3-oxosuccinate by isopropylymalate dehydrogenase encoded by *leuB* gene and in the presence of aminotransferase, the end product leucine is synthesized. The presence of leucine affects the activity of both isopropylmalate synthase and aminotransferase activity, i.e., the amount of leucine regulates the expression of these two enzymes. The gene *leuB* was targeted for mutation, as its expression is not regulated by the amount of leucine availability. And also, the commonly used cloning strain HB101 of *E. coli* is also *leuB* deficient. This allows for initial characterization of the *leuB* plasmid before being transformed into *Brucella*. Complementation with a medium copy number plasmid borne *trans* acting intact *leuB* gene will not be toxic as the enzyme acts only on an intermediate step and not on initial or end step of biosynthesis.

The *cre-lox* system has been used successfully for creating marker-less mutants without antibiotic resistance genes in various bacteria (14). This technology uses two co-directional recognition sequences called *loxP* sites, which in the presence of a recombinase enzyme will excise the DNA region in between them (17). The recombinase enzyme (*cre*) is derived from P1 phage (17). In this work, *cre-lox* technology was used to produce an unmarked *leuB* mutant in the cattle vaccine *B. abortus* strain RB51. The resultant *leuB* auxotroph cannot grow in leucine deficient minimal medium, an

environment similar to phagosomes of macrophage (7). Complementation of the *leuB* auxotroph with a plasmid carrying the wild type *leuB* gene allows its survival on leucine deficient minimal medium and *in vivo* nutrient limiting conditions thus providing selective pressure for maintenance of the plasmid.

The broad range cloning vector pBBR1MCS and its derivatives are stable inside *Brucella* species under both *in vitro* and *in vivo* conditions and so they are used to over-express genes of interest (8, 31). Lately, the improved derivatives of the pNS group of plasmids are used to over-express candidate antigens in *Brucella* and other bacterial species (24, 26, 28). For this research, an intact *leuB* gene replaced the antibiotic resistance cassette in pNSGroE to act as a plasmid marker in the complemented RB51*leuB*. The new plasmid without antibiotic resistance marker was named pNS4. The new plasmid possesses all the characteristics of pNS group of vectors and has the added advantage of having a non-antibiotic marker. Green fluorescent protein (GFP) has been used as a visual marker as well as a model antigen in our experiments (25, 27, 28). To elucidate the ability of the new plasmid pNS4 to express foreign antigens, GFP was used as a model to clone and detect expression inside strain RB51*leuB*.

J774.A1 murine macrophages have been used to conduct cell culture infection experiments involving *Brucella* and to detect GFP expression after infection (28). Green fluorescence can be detected in macrophages infected with *Brucella* carrying a plasmid over-expressing GFP when visualized using a confocal microscope (28). The protection efficiency of a candidate vaccine can be elucidated under laboratory conditions in a mouse model against a virulent strain challenge. Under field conditions, strain RB51 vaccine is used to immunize heterogeneous population of animals. To validate the

efficacy of our vaccine under laboratory conditions, using the out-bred mouse strain CD1 (12) would provide a outbred population similar to field conditions. The ability to over-express and elicit antigen (GFP) specific antibodies can be detected by analyzing the serum of mice using immunoblotting.

The *leuB* auxotroph of RB51 vaccine strain can be used as an environmentally safe vector to over-express homologous and heterologous antigens without concern for potentiating the spread of antibiotic resistance markers.

2. Materials and Methods

2.1. Bacterial strains, plasmids, media, and growth conditions.

Escherichia coli strain HB101 was kindly provided by Dr. Z. Young in the Department of Biological Sciences at Virginia Tech (Blacksburg, VA). The *B. abortus* vaccine strain RB51 was from our culture collection and made competent as described earlier (15). Plasmids used in this study are described in Table 1. Bacteria containing different plasmids were grown in presence of antibiotics at 5-10 μg/ml of gentamicin or 100 μg/ml of ampicillin or kanamycin. Z-competent *E. coli* DH5α (Zymo Research, Orange, CA) were prepared as per the manufacturer's instructions. *Brucella* minimal medium (BMM) was prepared as described by Plommet (19) or trypticase soy (TS) broth or agar (Difco); all incubations were done at 37^{o}C in the presence of 5% CO_2. Standard laboratory procedures recommended by Centers for Disease Control were followed while handling live *Brucella* in a Bio-safety level 3 facility of the Virginia-Maryland Regional College of Veterinary Medicine.

2.2. Construction of pNS4:

The *leuB* gene of the strain RB51 along with its own promoter (1412 bp) was amplified by PCR using the primers (*leuB*Forward, 5'GGG-AAG-CTT-GGG-TCT-AGA-AGT-TTC-GCT-CGC-GGT-GAG-TGG-CGA 3' and *leuB*Reverse, 5'GGG-ACT-AGT-TCA-GGC-CGA-AAG-TGC-CTT-GAA3'). The "origin of replication" (1700 bp) and 259 bp expression segment (*Brucella gro*E promoter + multiple cloning site + 6His tag) of the plasmid pNSGroE were amplified using the primers as described before as they have cloning sites as well as the minimal sequence necessary for plasmid replication (28); the *groE* promoter is up regulated following *Brucella* uptake into macrophages (31). After the restriction enzyme digestion, the 3 fragments were purified and ligated to form plasmid pNS4; note that this plasmid does not have an antibiotic resistance gene (Fig. 3). The *leuB* gene acts to complement any *leuB* auxotrophic strains carrying the plasmid under leucine limiting conditions i.e. minimal medium or inside the macrophage. Green fluorescent protein (GFP) gene, which is used as a model heterologous antigen, was cloned into the MCS of pNS4 using *BamHI* and *XbaI* sites.

2.3. Construction of the suicide plasmid pLGL and strain RB51*leuB.*

The *leuB* gene of *B. abortus* strain RB51 was amplified as two separate fragments of 750 bp and 450 bp. The first fragment contains 300 bp upstream of the *leuB* gene and was cloned using the primers (*leuB*I Forward 5' GGG GAA TTC AGT TTC GCT CGC GGT GAG TGG 3' and *leuB*I Reverse 5' GGG GGA TCC ATG ATT TCC TTC GGT TCG CCG 3'). The second fragment has 450 bp of the *leuB* gene and was amplified using the primers (*leuB*II Forward 5' GGG GGA TCC TAT GCT GGC TGA TGC TGG

CGG 3' and *leuB*II Reverse 5' GGG AAG CTT TCA GGC CGA AAG TGC CTT GAA 3'). These amplicons deliberately delete 216 bp of the *leuB* in order to minimize any reversal of the deletion during subsequent auxotroph growth. The intermediate construct pGL51 contains a 1200 bp amplicon containing a disrupted *leuB* gene cloned into vector pGEM3Z. The *aac*C1 gene coding for gentamicin resistance flanked by *loxP* sites was cloned as a single *BamHI* fragment from the plasmid pUCGm*lox* (20). This fragment was cloned into the *BamHI* site of the disrupted *leuB* gene of the intermediate construct to produce the final suicide plasmid pLGL.

The suicide plasmid pLGL was introduced into *B. abortus* strain RB51 by electroporation as described by McQuiston *et al.,* (15). The transformants (RB51Gmlox) were plated on TSA plates containing gentamicin and subsequently screened on TSA plates containing ampicillin. Amplification of the interrupted *leuB* gene carrying the *loxP*-flanked gentamicin was performed using *leuB* specific primers to verify the alteration in chromosomal *leuB* following recombination.

To create the unmarked deletion mutant, the plasmid pCM158 (Kan^R), which carries the "*cre"* gene encoding a site-specific recombinase, was transformed into competent *Brucella* strain RB51Gmlox. The recombinase catalyses the homologous recombination between the two *loxP* sites and thus excises the gentamicin resistance gene leaving one *loxP* sequence (Fig. 2A). The transformants were plated on TSA containing kanamycin to select for uptake of "*cre*" expressing plasmid and then individual kanamycin resistant clones were plated on a non-selective media to screen for segregation of the plasmid pCM158. Rough strain RB51 took one passage to clear pCM158 on non-selective TSA to produce segregants (14). Confirmation of the clearance of pCM158 was

done by replica plating the colonies on a kanamycin containing TSA plate and checking for loss of kanamycin resistance gene using specific primers in a PCR reaction.

2.4. Southern hybridization.

Southern hybridization was performed as described previously (11). Briefly, genomic DNA from the wild type and the mutant strains was extracted using Qiagen DNeasy tissue kit (Cat No. 69506). *B. abortus* DNA was digested with *KpnI* and the fragments were separated electrophoretically in an agarose gel and transferred to nitrocellulose membrane. The suicide plasmid pLGL and the Cre expression plasmid pCM158 were labeled with digoxigenin (DIG High prime DNA labeling kit, Roche Applied Science, Penzberg, Germany) and used as probes in two separate blots.

2.5. Growth curve experiment

A single colony of a particular *B. abortus* strain was inoculated in liquid minimal medium and grown for 72 h at 37°C and 200 rpm to create a starter culture that was used to inoculate the minimal medium and adjusted to 10-12 Klett Units (KU). At different time points the KU were measured using Klett-Summerson colorimeter (New York, NY) and corresponding CFUs were determined by plating serial dilutions onto TSA agar followed by incubation at 37°C for 4 days in the presence of 5% CO_2.

2.6. Macrophage experiments.

Murine J774.A1 macrophage cells (American Type Culture Collection, Manassas, VA) were incubated in a DMEM media (Cellgro, Mediatech Inc, Herndon, VA)

containing 10% Fetal Bovine Serum (FBS) for 24 h at 37°C in 5% CO_2 on a 6 well plate to make them adherent on a cover slip. A 48 h culture of the pNS4/GFP-complemented strain RB51*leuB* was resuspended in PBS and used to infect the macrophages at 1:100 multiplicity of infection (macrophage/bacteria). After 45 min, the macrophages were washed 3 times with PBS and then incubated in DMEM media containing 200 μg/ml of streptomycin and 200 I.U./ml of penicillin. At 24, 36 and 72 h post-infection, the cover slip was washed, fixed in formalin and mounted on glass slides. The slides were observed under Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Thornwood, NY) under fluorescence mode to detect expression of GFP.

2.7. Experiments in CD1 mice.

The protective efficacies of the *B. abortus* RB51*leuB* strain and the *leuB*-complemented strain were evaluated using 5 to 6 weeks old female CD1 mice (Charles River Laboratories, Wilmington, MA). Four groups of 10 mice per group were vaccinated intraperitoneally with ~ 3-5 x 10^8 CFU in 100 μl inoculum of either strain RB51, RB51*leuB*, RB51*leuB*/pNS4, or RB51*leuB*/pNS4/GFP. Another group of 10 mice were vaccinated with saline to serve as negative control. Three mice from each group were bled 5 weeks post vaccination for harvesting serum. At 6 weeks post vaccination, all groups of mice were challenged intraperitoneally with 4x10^4 CFU of *B. abortus* 2308. At 2 weeks post challenge, mice were euthanized by CO_2 asphyxiation and their spleens were recovered, homogenized, serially diluted and plated on TSA plates to estimate CFUs. All procedures involving mice were performed following Virginia Polytechnic Institute and State University's animal care committee recommendations (IACUC).

2.8. ELISA.

Serum antibody (IgG isotypes) responses of the mice to the *B. abortus* RB51 strains were determined by ELISA as described by Vemulapalli *et al.,* (31).

2.9. Immunoblot.

Green fluorescent protein (GFP) was extracted by nickel column purification from recombinant *E. coli* (Mach1) expressing plasmid pRSETA-GFP (source: our culture collection). The purified GFP was electrophoresed in a 12% denaturing PAGE and transferred onto a 0.45 μm nitrocellulose membrane (Trans-Blot, BIO-RAD Laboratories, Hercules, CA). Using Mini-PROTEAN II multiscreen apparatus (BIO-RAD, Hercules, CA), serum samples from individual mice of all the groups were used as primary antibody (1:50) and incubated with the membrane overnight at room temperature. Mouse monoclonal anti-GFP serum (1:1000; BD Biosciences Clontech, Palo Alto, CA) was used as a positive control. Peroxidase conjugated anti-mouse serum was used as the secondary antibody. CN/DAB with stable peroxidase substrate (PIERCE, Rockford, IL) was used as per manufacturer's instructions to detect the protein expression.

2.10. Statistical analyses.

The splenic recovery of CFU/spleen of the challenge strain *B. abortus* 2308 was subjected to analysis of variance; their means and variances were compared by Tukey's range procedure (Honestly significant difference) method.

3. Results

3.1. Characterization of *B. abortus* RB51Gm*lox* and RB51*leuB*.

Upon introduction of the suicide plasmid pLGL into strain RB51 several clones whose *leuB* gene was interrupted by the *loxP-aacC1-loxP* cassette were selected on TSA plates containing gentamicin and then screened for loss of ampicillin resistance. The interruption of the *leuB* gene was confirmed by PCR using the *leuB* forward and reverse primers (Fig. 2B). The *leuB* deficient transformant strain RB51 grew on the leucine-supplemented plates but not on leucine deficient *Brucella* minimal medium (BMM) agar. The loss of "*cre*" plasmid, pCM158 was demonstrated by the inability of the strains to grow on TSA plates containing kanamycin. PCR amplification was then used to confirm the excision of the *aacC1-loxP* fragment. Lanes 1 and 5 (Fig. 2B) show the size of the native *leuB* gene from two different *B. abortus* clones amplified using *leuB* forward and reverse primers; lanes 2 and 6 show the increase in size of the amplicon because of the replacement of the wild type gene with (*leuBI-loxP-aacC1-loxP-leuBII*) fragment. Lanes 3 and 7 reveal the resolution of the *leuBI-loxP-leuBII* gene as a result of the crossover between the directly repeated *loxP* flanking sites mediated by the Cre recombinase. No amplicons were observed following PCR amplification using primers specific for the kanamycin gene in the plasmid pCM158; both positive and negative controls were used. Thus it appears that pCM158 was lost, as expected, by segregation from the final *leuB* auxotroph. Moreover, when the entire plasmid pCM158 was used as a probe, no signal was detected in a Southern blot and confirmed the absence of the plasmid in the final auxotroph (results not shown). To validate that the Cre mediated deletion had taken place only in *leuB* gene, a Southern blot analysis using the entire suicide plasmid pLGL showed no signal other than in the *leuB* gene (Fig. 2C).

3.2. Growth curves.

The doubling time of the *leuB* complemented auxotroph strain RB51*leuB* in minimal medium was observed to be approximately about 6-8 hrs. Leucine deficient BMM did not support the growth of the *leuB* auxotroph. Complementation of the *leuB* auxotrophs with pNS4 restored their growth in leucine deficient BMM. Expression of GFP in *B.abortus*/pNS4/GFP did not affect the strain's ability to be complemented with *leuB* (Fig. 4).

3.3. Expression of GFP inside macrophages:

J774.A1 macrophages infected with RB51*leuB* containing the *leuB* complementing plasmid pNS4/GFP appeared green when observed under fluorescent confocal microscopy. A representative picture of a macrophage containing RB51*leuB* expressing GFP at 36 h post infection is shown (Fig. 5).

3.4. Efficacy of the vaccine in CD1 mice.

The *leuB* auxotroph and the complemented auxotroph of strain RB51*leuB* were able to significantly protect the CD1 mice against a virulent *B. abortus* 2308 challenge (Fig. 6). There was no significant difference in the protection levels (i.e. splenic clearance) afforded by the *leuB* auxotroph, when the complemented *leuB* auxotroph and the complemented *leuB* auxotroph expressing GFP were compared to the mice vaccinated with the strain RB51. There was however, a significant difference in protection afforded between the mice vaccinated with any of the RB51 strains and the saline control ($P < 0.05$). There was no significant difference in protection levels observed between the

vaccine strain RB51 and the *leuB* auxotrophs of strain RB51 when Tukey's range procedure was used.

In a separate experiment, the *leuB* auxotroph and the complemented *leuB* auxotroph were cleared by 4 to 5 weeks from CD1 mouse spleens at the same rate as the parent vaccine strain RB51 (data not shown). The stability of pNS4/GFP and its ability to express antigen *in vivo* were demonstrated by showing that green fluorescence was found in all the *B. abortus* vaccine clones recovered from spleen 4 weeks post vaccination i.e. prior to clearance. In addition, plasmid extraction of those green fluorescent colonies showed that their 4.1 kb size was consistent with pNS4/GFP as determined by restriction digestion.

3.5. Serum antibody response in CD1 mice.

Specific antibody response (IgG1, IgG2a and whole IgG sub-isotypes) showed a bias towards Th1 type (IgG2a) immune response against all the RB51*leuB* vaccine strains (results not shown). Western blot analysis using purified GFP revealed that only the mice vaccinated with strain RB51*leuB* complemented with plasmid pNS4/GFP expressed GFP specific antibodies (Fig. 7).

4. Discussion.

Because the USDA approved vaccine strain RB51 is a good inducer of a Th1 response (3, 31), we have investigated the strain as an antigen delivery platform for vaccination against intracellular pathogens (29, 32). Recently, we have demonstrated both protective cell-mediated immunity (CMI) and antibody-mediated immunity against

Neospora caninum (NC) in mice using strain RB51 as a platform for expression of NC antigens (21, 22). We have also refined the broad host range plasmid pBBR1MCS to allow for strong expression of heterologous antigens as well as their purification from *Brucella spp*. (28). Since the US Food and Drug Administration discourages the use of antibiotic resistance markers for antigen expression in live vaccines (1, 13, 33), the plasmid pNS4 was constructed without an antibiotic resistance gene. We have successfully used this plasmid not only to complement the leucine deficiency of the strain RB51*leuB* but also to over-express a heterologous antigen (GFP) both *in vitro* (pure culture) and *in vivo* (in mice). The stability of the plasmid pBBR1MCS in *Brucella* is well documented (32). The refined new group of pNS plasmids (28) compared to its parent plasmid pBBR1MCS, fares better in stability inside *Brucella* under non-selective conditions *in vitro* and *in vivo*. Even after 11 subcultures in an enriched media (non-selective condition), we were able to recover the plasmid pNS4 from 10 randomly selected colonies of *leuB* deficient strain RB51. When viewed under ultraviolet light all the colonies of strain RB51*leuB* complemented with pNS4GroE/GFP recovered from CD1 mice spleen 4 weeks after vaccination were fluorescent. Combined with the green fluorescence observed in macrophages infected with *B. abortus* RB51*leuB*/pNS4/GFP (Fig. 5), this strongly suggests the pNS4 was able to express a heterologous antigen inside RB51*leuB* following immunization of mice.

In this report, we successfully utilized *cre-lox* technology to create a leucine auxotroph of *B. abortus* strain RB51. *Brucella* now joins the list of other Gram-negative bacteria (14) that can be mutated using the *cre-lox* approach. Other techniques have been employed to create mutants of *Brucella* include allelic exchange mediated by suicide

plasmids (5, 6), allelic exchange followed by counter selection against sucrose resistance mediated by the *sacB* gene (10) and transposon directed mutagenesis (18). The advantage of using the *cre-lox* technology is the enzyme-mediated loss of the regions targeted by *loxP* sites result in a deletion marked by a single *loxP* site. This will allow the subsequent use of additional pair of *loxP* sites to make additional mutations in the bacterial genome (14). In addition, it also should be recognized that the *cre-lox* technology can be used to direct the introduction of single copy of a homologous or heterologous gene for chromosomal expression purposes in which a high copy number plasmid encoded gene produces a toxic effect or if the plasmid is not stable inside the bacteria.

The protective efficacy of the RB51*leuB* vaccine strain and the *leuB*-complemented version in CD1 mice was as good as that found for strain RB51 vaccine in BALB/c mice (Fig. 6). Unlike previous experiments, we used the CD1 strain of mice, an outbred strain (12), to more closely resemble outbred genetic backgrounds subjected to vaccination in field conditions, e.g. cattle. Both the leucine auxotroph and the complemented version of *B. abortus* strain RB51 were cleared in CD1 strain mice at the same rate as they were in inbred BALB/c mice (data not shown). Moreover, crystal violet staining of *Brucella* isolates from spleens 2 weeks following challenge revealed that all the isolates were smooth and confirmed the clearance of RB51*leuB* strain used for immunization. Serum collected from the CD1 mice 5 weeks post vaccination (before challenge) shows that the predominantly IgG2a response developed is biased towards a Th1 type. Immunoblotting using purified GFP as antigen showed that the mice vaccinated with strain RB51*leuB*/pNS4/GFP developed a GFP specific antibody response

(Fig. 7). This observation suggests that in principle, any protective homologous or heterologous antigen can replace the GFP gene and that the RB51*leuB* strain over-expressing the antigen most likely would induce a protective response against both *B. abortus* and perhaps against other infectious agents requiring a Th1 response.

5. Conclusion:

Over-expressing protective antigens in strain RB51 is a proven methodology for developing multivalent vaccines (31, 32). The new vaccine strain RB51*leuB* along with the complementing plasmid pNS4 developed in this study would be a good combination as an environmentally safer multivalent live vaccine. This new RB51*leuB* vaccine strain could be used to help protect livestock against brucellosis and other infectious diseases.

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TABLE. 1. Description of plasmids used in this study

Plasmid	Description	Source or reference
pNSGroE	Expression vector, chloramphenicol resistant	(28)
pNS4	*leuB* gene of *B. abortus* replaced the chloramphenicol resistance gene in pNSGroE	This study
pGEM-3Z	Cloning vector, Amp^r	Promega
pGL51	1200bp of *leuB* gene was amplified by PCR and cloned into the *HindIII* and *XbaI* sites of pGEM-3Z, Amp^r	This study
pUCGm*lox*	pUC18-based vector containing the lox sites flanked *aacC1* gene, Amp^r, Gm^r	(20)
pLGL	pGL51 plus the *aacC1* gene flanked by *loxP* sites cloned into the *BamHI* site within the mutated *leuB* gene, Amp^r, Gm^r	This study
pCM158	"Cre" expression plasmid, Kan^r	(14)
pNS4/GFP	Green fluorescent protein gene cloned in the multiple cloning site of pNS4	This study

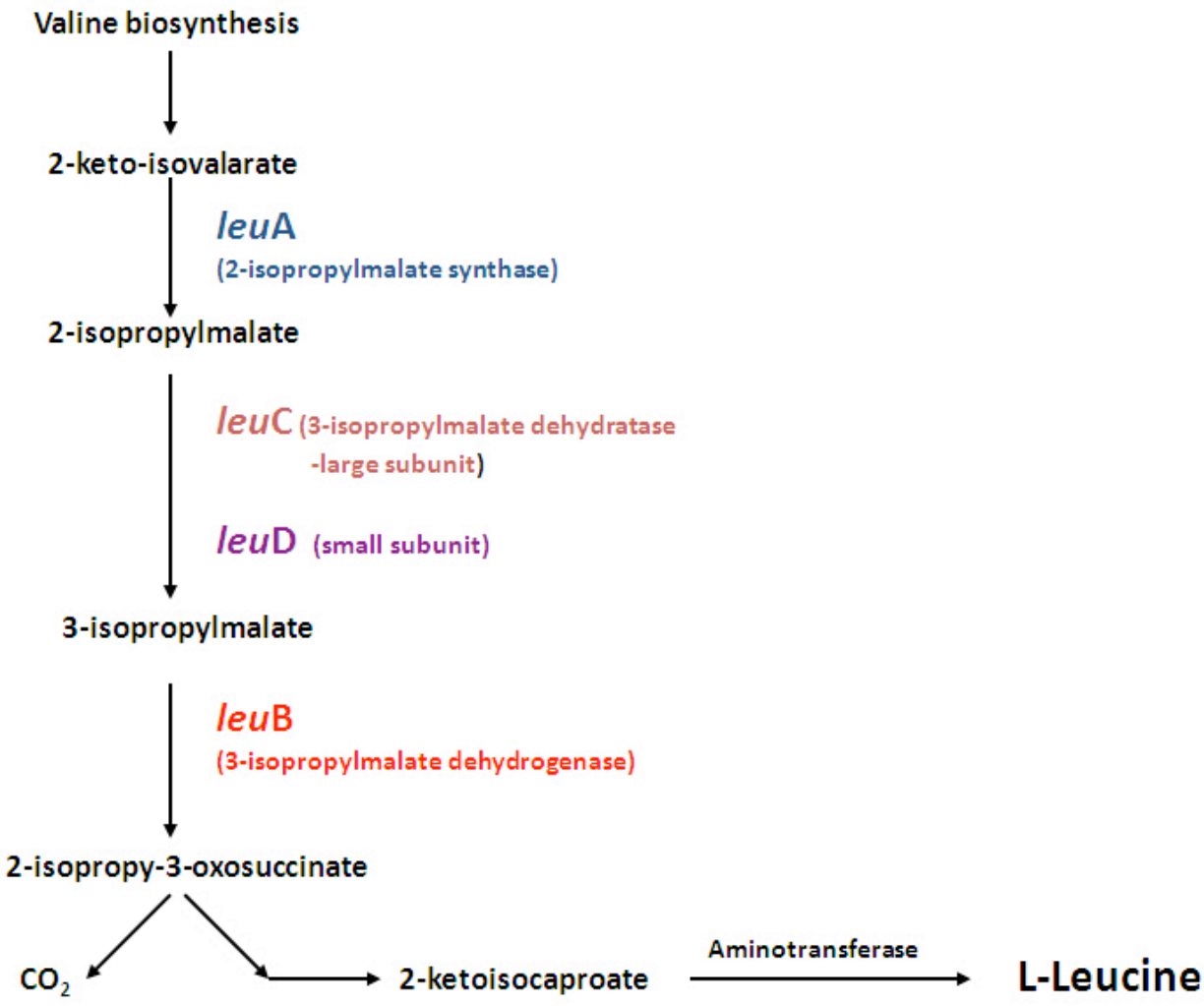

Fig. 1 Leucine biosynthesis

(Reference: http://patricpathways.vbi.vt.edu)

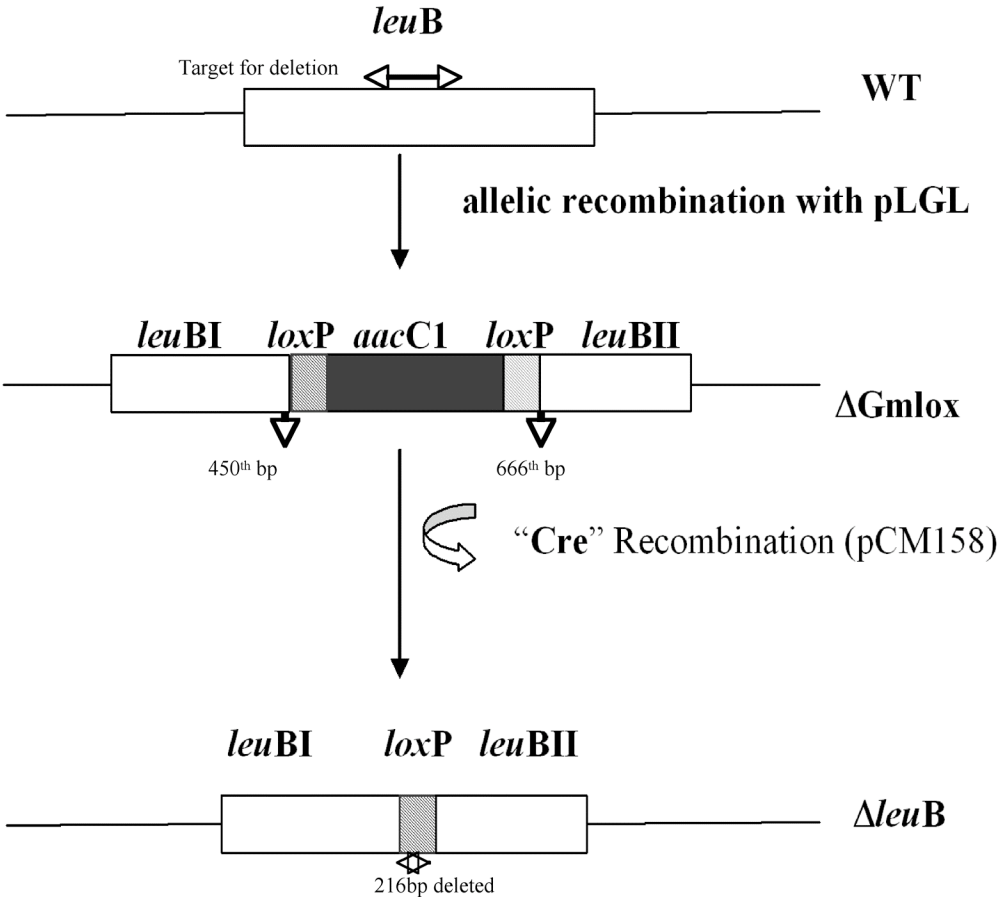

Fig. 2A. Illustration of the recombination events. The *leuB* gene is disrupted between 450th and 666th base pairs and the final unmarked mutant lacks 216 bp. WT - Wild type, ΔGmlox - intermediate marked mutant, Δ*leuB* – unmarked *leuB* mutant.

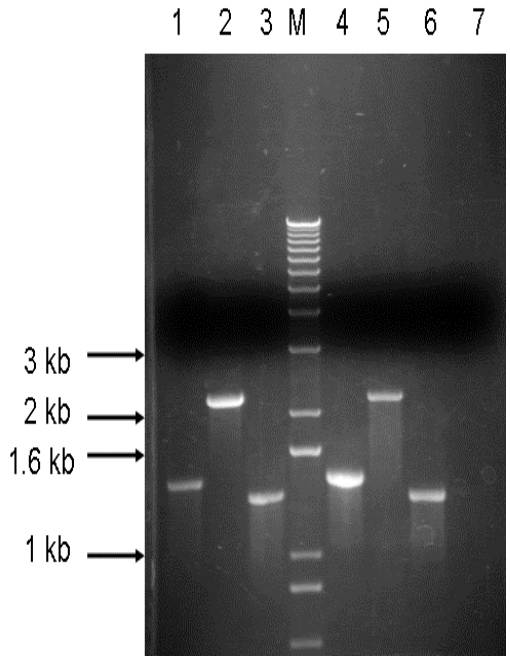

Fig. 2B. PCR amplicons of the *leuB* gene from different strains. Lane. Strain (Size). M. 1kb plus DNA Ladder, 1&4. RB51 (1.4kb), 2&5. RB51Gm*lox* (2.2kb), 3&6. RB51*leuB* (1.3kb), 7. Negative control

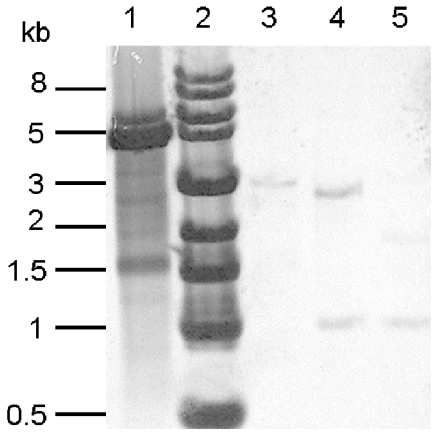

Fig. 2C. Southern hybridization of *Kpn*I digested genomic DNA using pLGL as a probe to demonstrate that the integration occurred only at the *leu*B site of the genome. Lane, DNA (expected band size(s)): 1, pLGL (4.8 kb); 2, Molecular mass standards; 3, RB51 (3 kb); 4, RB51Gm*lox* (2.7 kb and 1 kb); 5, RB51*leu*B (1.8 kb and 1 kb).

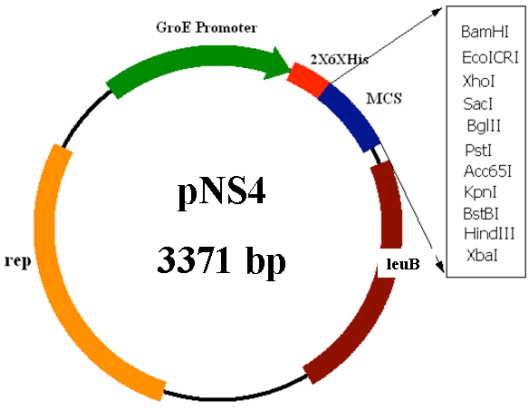

Fig. 3. Illustration of plasmid pNS4

rep – Origin of replication

MCS – Multiple cloning site

2x6xHis- 2x 6Histidine tag

leuB – intact *leuB* gene with its promoter

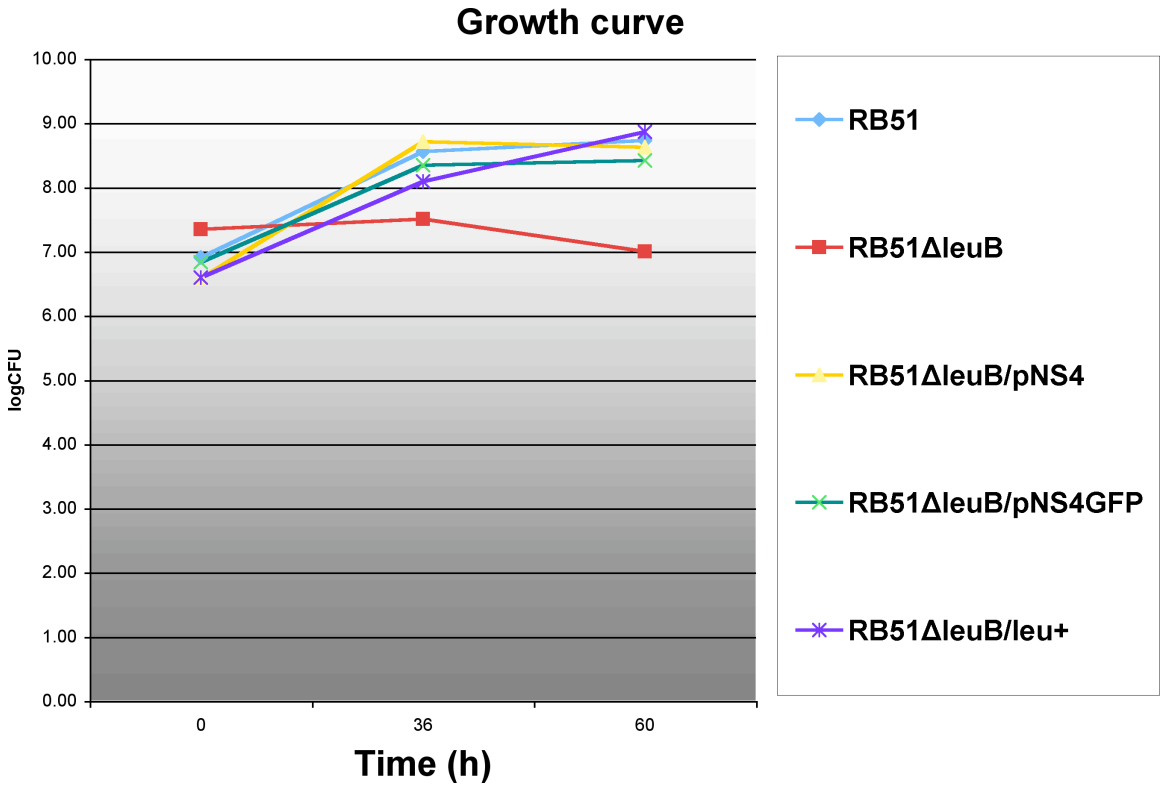

Fig. 4. Growth based on CFU of the different strains RB51, RB51*leu*B, RB51*leu*B/pNS4, RB51*leu*B/pNS4/GFP, RB51*leu*B/leu+ (leucine supplemented) at different time points when grown in a leucine deficient *Brucella* Minimal Medium.

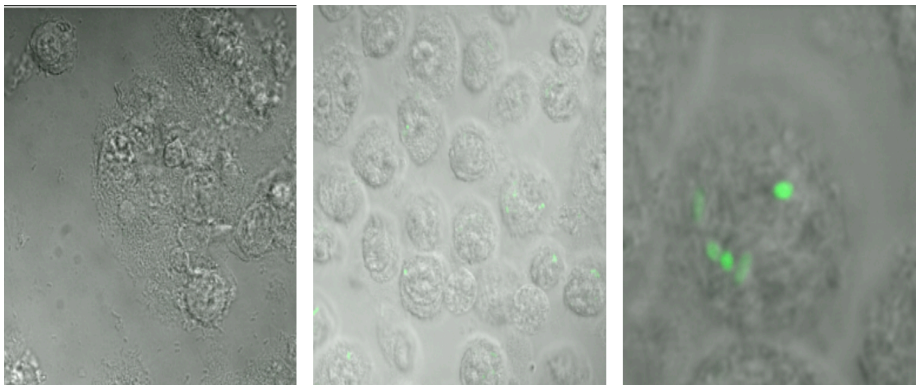

Fig. 5. Confocal microscope image of *Brucella* infected macrophages. Panel. 1. Uninfected J774.A1 macrophages (Negative control). Panel. 2. J774.A1 macrophages infected with a *leu*B mutant of strain RB51 expressing GFP viewed under confocal microscopy at 36 h post-infection. Panel. 3. A single macrophage containing GFP expressing RB51*leuB* strains under higher magnification at 36 h post-infection.

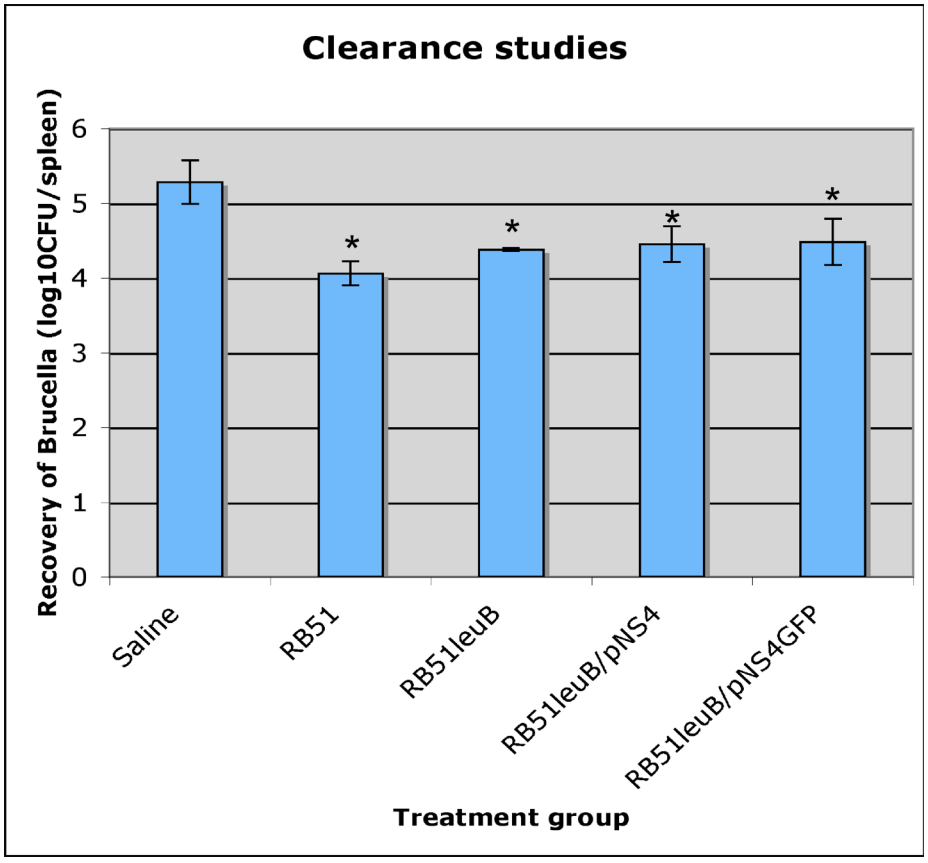

Fig. 6. Clearance of the challenge strain *B. abortus* 2308 in CD1 mice at 2 weeks post-challenge. Mice (n=10) vaccinated with the *leu*B mutant and the complemented RB51*leu*B strains were able to clear the challenge strain *B. abortus* 2308 significantly faster compared with the mice vaccinated with saline *(P* < 0.05). No significant difference was observed in the clearance rates of challenge strain in the mice vaccinated with auxotrophic vaccine strains and the standard strain RB51 and also within each other when ANOVA and Tukey's range procedure was used. (* Indicates significant difference, $P < 0.05$)

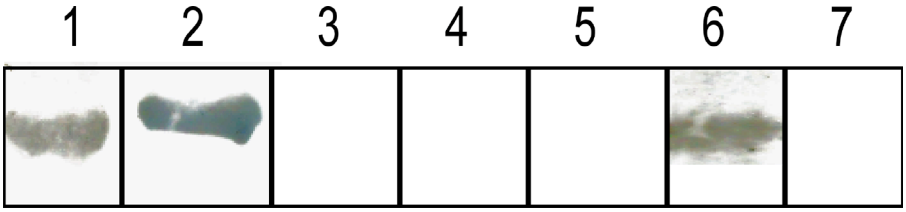

Fig. 7. Immunoblot of GFP. A crude extract (7 μg) from *E. coli* expressing GFP was separated by 10% SDS-PAGE & transferred to nitrocellulose membrane. The membrane was split into individual lanes and treated with immunized mouse or control sera. Lane(s), Serum group: 1, strain RB51*leu*B/pNS4/GFP (Band size – 27 kDa); 2, Molecular mass standards; 3, strain RB51; 4, strain RB51*leu*B; 5, strain RB51*leu*B/pNS4; 6, Anti-GFP serum (positive control); 7, no serum (negative control). Results shown are those from the 25-30kDa region of the gel; no other positive reactions were seen on the lanes incubated with the serum from mice immunized with GFP.

Chapter 3

Over-expression of homologous antigens in *Brucella abortus* strain RB51*leuB* protects mice against a virulent *B. suis* challenge

Rajasekaran, P., Naveen Surendran, Mohamed N. Seleem, Nammalwar Sriranganathan, Gerhardt G. Schurig, and Stephen M. Boyle.

(Prepared for submission to Infection and Immunity)

Abstract

The infection by members of the Gram-negative bacterial genus *Brucella* causes brucellosis in mammals. *B. abortus* strain RB51 is the officially approved vaccine being used in USA and several other countries to control bovine brucellosis. There is no effective vaccine available that is approved for use in swine against brucellosis caused by *Brucella spp*. Earlier, using a chloramphenicol resistance plasmid, we showed that over-expression of *Brucella* derived antigens (SOD and O-side chain) in strain RB51 confers cross protection against *B. melitensis* infection in mice. Recently, we constructed an environmentally safe, antibiotic resistance free over-expression system using a leucine auxotroph of strain RB51 as platform and successfully employed the system in mice with GFP acting as a model antigen. Using the same strain RB51*leuB* as a base, RB51*leuB*/SOD, RB51*leuB*/SOD/L7/L12 and RB51*leuB*/SOD/WboA were constructed to over-express the antigens; SOD alone, SOD with ribosomal protein L7/L12 and SOD with O-side chain (in cytoplasm) respectively. The ability of these vaccine candidates to protect against a virulent *B. suis* challenge were evaluated in a mouse model. All vaccine groups protected mice significantly ($P < 0.05$) when compared to the control group. Within the vaccine groups, the mice vaccinated with RB51*leuB*/SOD/WboA were

significantly better protected than those that were vaccinated with either RB51*leuB*/SOD or RB51*leuB*/SOD/L7/L12. These results suggest that *Brucella* antigens can be over-expressed in strain RB51*leuB* platform and could elicit protective immune responses against brucellosis. Since the platform system is antibiotic resistance free, it complies with regulations and therefore could be used to develop safer multivalent vaccines.

Key words: RB51*leuB*, *Brucella suis*, SOD, WboA and antibiotic resistance

INTRODUCTION

Swine brucellosis is primarily caused by *Brucella suis*, a facultative intracellular Gram-negative bacterium. Infection in swine causes abortion, infertility and overall low productivity in farms thus causing economic losses. Humans contract brucellosis either by consumption of infected raw or uncooked milk and milk products or by coming in contact with infected animals through occupational or recreational exposures (1). As a zoonotic pathogen, *B. suis* infection in human causes flu like symptoms and undulant fever (1). A report of the Animal and Plant Health Inspection Service (APHIS) under United States Department of Agriculture (USDA) states that, as of august 2009, except the state of Texas, all commercial swine farms in US are brucellosis free (http://www.aphis.usda.gov/animal_health/animal_dis_spec/swine/images/br_status_map .jpg). Though the disease is not widely prevalent in domestic swine population of the

United States and in some other developed countries, it is still endemic in feral swine and wild animal populations and acts as a zoonotic reservoir for spread to domestic animals and humans (1, 14, 28, 35). According to Food and Agriculture Organization (FAO), there is no approved vaccine available for humans against brucellosis. Development of an effective vaccine, that could be used in livestock especially in swine, against *B. suis* infections would help minimize the incidence of human infections. A Chinese vaccine *B. suis* strain S2 (biovar 2) has been used with reasonable success in swine, small ruminants and cattle as a prophylaxis against brucellosis but, needs more experimental data to verify its efficacy before being recommended for wider field use (Guidelines for coordinated human and animal brucellosis surveillance *Robinson, A., Animal Production and Health Div. FAO* 2003). Since strain S2 is a smooth strain, differentiating vaccinated and infected animals will be difficult as the serological diagnostic tests are based on antibodies to O-polysaccharide region of the lipopolysaccharide of smooth strains (12, 26). In an attempt to make strain S2 into a rough strain that can be differentiated from the smooth virulent strains, the *wbkC* gene was replaced by a chloramphenicol resistance gene (12). Introducing antibiotic resistance genes containing recombinant bacteria as a vaccine into ecosystem is discouraged and strictly regulated by United States Food and Drug Administration (FDA) (61 FR 68269) (2). Development of a vaccine strain that would not interfere with diagnostic tests and also complies with FDA regulations would be the desired infection intervention strategy in endemic areas.

B. abortus strain RB51 is an USDA approved vaccine used against bovine brucellosis (24). As a stable rough derivative of the smooth *B. abortus* strain 2308, strain RB51 lacks the ability to produce the O-side chain associated with the lipopolysaccharide

region of the cell envelope and thus does not interfere with diagnostic tests (23). As a live attenuated vaccine, strain RB51, after phagocytosis, delivers antigens at intracellular level during that process, and thereby can be used as a vaccine vector for delivering antigens from pathogens that need cell mediated immunity (CMI) for protection (20, 22, 29, 31, 34). But the vaccine strain RB51 was not able to protect swine against a virulent *B. suis* challenge (27). Earlier we have showed that over-expression of homologous antigens on strain RB51 enhanced the protection efficacy of the vaccine against *B. abortus* and *B. melitensis* challenge under experimental conditions (29, 33). Hence, over-expressing protective antigens on strain RB51 platform is expected to enhance its protective efficacy against a *B. suis* challenge.

In our efforts to develop the next generation brucellosis vaccines using strain RB51 as a platform, we constructed the environmentally safe leucine auxotroph of strain RB51 to over-express antigens without using antibiotic resistance marked plasmids (19). We successfully demonstrated the over-expression of a model antigen (GFP), in strain RB51*leuB* platform using the antibiotic resistance free plasmid pNS4 both *in vitro* and in mouse model (19). The leucine auxotroph derived from strain RB51 can be used to develop multivalent vaccines by over-expressing protective antigens derived from either *Brucella* (homologous) and/or other pathogens (heterologous) (19).

Previous work in our research group shows that complementation of the gene *wboA* (encoding glycosyl transferase) in *B. abortus* strain RB51 elicits a protective immune response in mice against *B. melitensis* strain 16M challenge (29). The glycosyl transferase encoded by gene *wboA* catalyses the synthesis of the highly immunogenic O-side chain region of *Brucella* lipopolysaccharide (LPS) (32). The Cu/Zn superoxide

dismutase (SOD) encoded by gene *sodC* encodes another antigen of *Brucella*, also elicits an enhanced protective immune response against *B. abortus* upon over-expression in strain RB51 (29, 33). Over-expression of O-side chain along with SOD in strain RB51 enhanced the vaccine's efficiency by cross protecting against a *B. melitensis* but without altering the attenuation characteristics and rough phenotype of the parental vaccine strain (29, 32). The *Brucella* ribosomal protein L7/L12 when administered as DNA vaccine along with SOD or as a purified protein by itself has been shown to elicit protective immunity against *B. abortus* challenge (10, 11, 16). Previous work in our laboratories showed that when gene *rplL* (encoding ribosomal protein L7/L12) was over-expressed in strain RB51 platform, mice were cross-protected against a *B. melitensis* challenge (Ashraf Omar, Ph.D. Dissertation, 2007, Zagazig University, Egypt).

Based on the above observations the genes *wboA* and *rplL* were over-expressed as two different constructs in the strain RB51*leuB* platform using the antibiotic resistance free plasmid pNS4. The antigenic SOD was co-expressed in both of the plasmid constructs to enhance the Th1 immune response as previous results show that combined expression augments protective immune response without increasing virulence characteristics of the vaccine strain (29, 36). While the genes *sodC* and *wboA* were expressed under their own promoters, the gene *rplL* was expressed from the *bla* (beta-lactamase) promoter on the plasmid. The advantage of using the leucine auxotroph strain RB51 and the *leuB* plasmid is that it complies with FDA vaccine regulations by not using antibiotic resistance plasmids. Here we demonstrate the protection afforded by various RB51*leuB* based vaccine constructs against a virulent *B. suis* challenge in a mouse model in an effort to develop an effective vaccine against swine brucellosis.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions.

The *Escherichia coli* strain HB101 and virulent *B. suis* strain 1330 used were from our culture collection at Virginia Tech (Blacksburg, VA). Z-competent *E. coli* HB101 (Zymo Research, Orange, CA) were prepared as per the manufacturer's instructions and was used for generation of plasmid constructs. The *B. abortus* strain RB51*leuB* from our culture collection was made competent as described earlier (13, 19). *Brucella* minimal medium (BMM) was prepared as described by Plommet (18); all incubations were done at 37°C in the presence of 5% CO_2. Standard laboratory procedures recommended by Centers for Disease Control ad Prevention (US) were followed while handling live *Brucella* in a Bio-safety Level 3 facility of the Virginia-Maryland Regional College of Veterinary Medicine.

Construction of strains RB51*leuB*/SOD, RB51*leuB*/SOD/L7/L12 and RB51*leuB* /SOD/WboA.

The antibiotic resistance free plasmid pNS4 (19) was used as the recipient construct to clone and express antigens of interest. The SOD gene with its promoter and a part of MCS region was excised as a 1.2 kb fragment from plasmid pBBSOD (33) using sites *SalI* and *XbaI*. The GroE promoter and the MCS region of the plasmid pNS4 (19) was excised using sites *SalI* and *XbaI*. The SOD fragment and the similarly excised pNS4 were ligated to generate the construct pNS4/SOD (Fig. 1A).

The region containing the gene *rplL* (encoding L7/L12) and the upstream region containing *bla* promoter was amplified from one of our plasmid collections using primers (Forward 5' CCC**AGATCT**AAGGGCCTCGTGATACGCCTA 3' and Reverse 5'

GGG**TCTAGA**TTACTTGAGTTCAACCTTGGC 3'). This 641 bp fragment also contains the "*bla*" signal sequence in between the promoter region and the gene. The amplified fragment was made to have sticky ends by using restriction enzymes *BglII* and *XbaI*. This fragment was ligated to the already made pNS4/SOD construct that was excised with *BamHI* and *XbaI* to produce the construct pNS4/SOD/L7/L12 (Fig. 1B). This construct could be used as a base to clone and express genes of interest under "*bla*" promoter and signal sequence using restriction sites *BamHI* and *XbaI* (replacing the gene L7/L12 with candidate gene). Any protective antigen can be cloned such that they are expressed by the *bla* promoter and would be translocated to the periplasmic space of *Brucella* by the *bla* signal sequence for better presentation to immune system (21). The *bla* promoter region includes the 174 bp upstream region of the gene and 105 bp of the actual *bla* gene containing the signal sequence to aid the translocation of antigen cloned downstream to the periplasmic space.

The 2.2 kb fragment containing genes *sodC* and *wboA* was excised from the plasmid pBB4SOD/WboA (29) by *XhoI* and *BamHI* digestion. This fragment was mixed with *SalI* and *BamHI* digested plasmid pNS4 (devoid of GroE promoter region) to generate the plasmid pNS4/SOD/WboA (Fig. 1C). All the three plasmid constructs pNS4/SOD, pNS4/SOD/L7/L12 and pNS4/SOD/WboA were transformed individually into RB51*leuB* by electroporation (1500V, 25μF, 200Ω) (13). After overnight recovery, the transformed bacteria were washed with saline once to remove the enriched media before being plated on leucine deficient minimal plates for selection of transformants.

Immunoblotting

Cell extracts of the candidate vaccine strains were prepared for western blotting using 10mM Tris-HCl buffer as described previously (33). The extracts were electrophoresed in a 10% Bis-Tris gel (Invitrogen Corporation, Carlsbad, CA) and transferred onto a 0.45 μm nitrocellulose membrane (Trans-Blot, BIO-RAD Laboratories, Hercules, CA). Goat anti-SOD serum (1:4000) (33) or Bru38 (32), rat monoclonal antibody to *Brucella* O-side chain (1:10) were used as primary antibodies. Peroxidase conjugated anti-goat or anti-rat sera (ICN pharmaceuticals Inc.) were used at 1:2000 as secondary antibodies. CN/DAB with stable peroxidase substrate (PIERCE, Rockford, IL) was used to detect the secondary antibody binding as per manufacturer's instructions.

***Brucella* RNA isolation, Reverse-Transcriptase PCR and real-time PCR**

Brucella RNA was isolated from bacterial cultures (~ 10^7 cfu) grown on BMM agar plates. The bacteria were harvested from plates by gentle scrapping and incubated in RNA protect (Qiagen) at room temperature for 5 minutes to stabilize the RNA before being harvested by centrifugation in a microfuge at 5000 rpm (2000 x G) for 3 minutes. The pelleted cells were resuspended in 1 ml of TRIzol (Invitrogen) and transferred into a tube containing micronium beads (FastRNA Pro Blue Kit, Q-BIOgene). *Brucella* were lysed on a Mini-bead beater 3110BX (Biospec products, OK) and then 0.1 ml of 1-bromo-3-chloropropane (Sigma) was added, mixed thoroughly, and incubated for 2-3 minutes at room temperature. The colorless upper phase containing RNA was carefully transferred into a fresh tube after centrifugation at 12,000 x G for 15 minutes at 4 °C. An equal volume of 70% ethanol was added to the fresh tube containing RNA and mixed well to avoid any precipitation. The whole mixture was later transferred to the column of the PureLink kit (Invitrogen) and final washing and elution steps were followed as per

manufacturer's instructions. The DNAse (Qiagen) treated RNA elution was used as a template to synthesize cDNA using the iscript cDNA synthesis kit (Bio-Rad laboratories) following the manufacturer's instructions.

For real-time PCR, the eluted RNA was used as template and iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad laboratories) was used as per the instructions supplied with the kit to produce PCR products and the signals were detected using iCycler iQ Real Time Detection System (Bio-Rad Laboratories).

Mice experiments

To assess the attenuation characteristics of the RB51*leuB* constructs in animal models, 4 weeks old female BALB/c mice (Harlan Laboratories, Indianapolis, IN) were received. All mice were allowed to acclimatize for two weeks in the animal facility before starting the experiment. Four groups of 15 mice/group were inoculated intraperitoneally with a 100 μl containing either strain RB51*leuB* (3.8×10^8 CFU/mouse), RB51*leuB*/SOD (9×10^8 CFU/mouse), RB51*leuB*/SOD/L7/L12 (8.2×10^8 CFU/mouse) or RB51*leuB*/SOD/*wboA* (2.8×10^8 CFU/mouse). The exact doses in CFU/mouse were calculated by plating the serial dilutions of candidate vaccine constructs retrospectively after vaccination. At 1, 3 and 5 weeks post vaccination, 5 mice from each group were euthanized and their spleens homogenized, serially diluted and plated on TSA plates. Blood was collected from these mice by cardiac puncture after the recovery of spleen and serum was collected by centrifugation of blood at 5000 rpm (2000 x G) for 10 minutes.

In a separate experiment, to evaluate the protective efficacies of the vaccine candidates, four groups of 5 mice per group were vaccinated intraperitoneally with ~ 3-8 x 10^8 CFU in 100 μl containing either strain RB51*leuB*, RB51*leuB*/SOD,

RB51*leuB*/SOD/L7/L12 or RB51*leuB*/SOD/*wboA*. Another group of 5 mice were vaccinated with saline to serve as negative control. At 6 weeks post vaccination, all groups of mice were challenged intraperitoneally with 1 x 10^5 CFU of *B. suis* strain 1330. At 2 weeks post challenge, mice were euthanized by CO_2 asphyxiation and their spleens were recovered, homogenized, serially diluted and plated on TSA plates to estimate CFUs. The lowest detection limit for bacterial burden was set at 20 CFUs/spleen.

All procedures involving mice were performed following Virginia Polytechnic Institute and State University's animal care committee recommendations (IACUC).

Splenocyte culture and quantification of interferon-γ and IL-4

In another mice experiment, at 7 weeks post inoculation, 5 mice/group that were administered either strain RB51*leuB*, RB51*leuB*/SOD, RB51*leuB*/SOD/L7/L12, RB51*leuB*/SOD/*wboA* or saline were euthanized and spleens were collected. A single cell suspension of spleen cells were made and the red blood cells were lysed using ACK solution (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA [pH 7.3]) as described (29). Cells were resuspended in RPMI media (GIBCO) containing 10% heat inactivated fetal bovine serum, L-glutamine and 5 x 10^{-5} M β-mercaptoethanol and plated in triplicates at 5 x 10^5cells/well in a 96-well flat-bottomed cell culture plate. The splenocytes were cultured in the presence of either heat killed RB51 cells (10^6 CFUs) or concanavalin A (0.5 μg), or no additives (control) for 5 days as described previously (29). After 5 days, the supernatant was collected after centrifugation at 1500 x g for 10 min and transferred to a new 96 well plate and stored at -80°C until ELISA was performed to determine the cytokine concentrations. IFN-γ and IL-4 cytokine levels were subsequently measured in duplicates for each mouse sample by indirect sandwich ELISA

using paired antibodies and standards from BD Pharmingen. The lower limits of detection for IFN-γ and IL-4 were 156 and 5 pg/ml respectively.

ELISA

Serum antibody (IgG sub-isotype) responses of the mice to the over-expressed antigens SOD and WboA were determined by indirect ELISA as described by Vemulapalli *et al.,* (29, 31). Briefly, *B. abortus* LPS or recombinant SOD at the concentration of 0.5 μg/well in 50 μl bicarbonate coating buffer were used to coat 96 well polysterene plates (Nunc Maxisorp) and incubated overnight at 4 °C. The plates were blocked with 2% bovine serum albumin (BSA) in PBS for 2 hours at room temperature. Pooled mice sera from 5 mice (1:100) were added in triplicates and incubated for 3 hours at room temperature. After three washes in PBS containing 0.05% Tween-20, isotype specific anti-mouse peroxidase conjugates (ICN pharmaceuticals Inc) were added and incubated for one hour at room temperature. After three washes, 100 μl of TMB substrate solution (KPL, Gaithersburg, MD) was added and incubated for 20 min in dark. The reaction was stopped by the addition of 0.18 M sulphuric acid (100 μl/well) and the absorbance of the color developed was measured at 450 nm.

Statistical analysis

The bacterial loads in spleen (CFU/spleen) of the vaccine strains and challenge strain *B. suis* 1330 were subjected to analysis of variance; their means and variances were compared by Tukey's range procedure (Honestly significant difference) method. The cytokines and IgG antibody levels were subjected to the same statistical analysis. ($P < 0.05$ was considered significant).

RESULTS

Over-expression of antigens SOD, L7/L12 and expression of O-side chain in strain RB51*leuB*.

Western blot analysis of antigen extracts of the candidate vaccine strains RB51*leuB*/SOD, RB51*leuB*/SOD/L7/L12, and RB51*leuB*/SOD/*wboA* screened against goat antiserum to SOD (33) showed that all the three strains over-expressed SOD (~ 19 kDa) (Fig. 2). The mRNA was isolated from strains RB51*leuB*/SOD and RB51*leuB*/SOD/L7/L12 and reverse transcribed to produce cDNA. Using the cDNA as template, a semi-quantitative PCR was done to analyze the over-expression of gene *rplL* (L7/L12) compared to a house keeping gene *ure1B* (encoding the beta subunit of urease I operon). The densitometer analysis showed that L7/L12 was over-expressed about 27% more in strain RB51*leuB*/SOD/L7/L12 compared to strain RB51*leuB*/SOD (Fig. 3). The net over-expression percentage was derived after normalizing with the expression of *ure1B* gene (3). The level of over-expression of ribosomal protein L7/L12 was also evaluated using real-time PCR, which showed that the construct RB51*leuB*/SOD/L7/L12 had 6 fold more expression of L7/L12 than the construct RB51*leuB*/SOD that has only the chromosomal copy of the gene *rplL* (Fig. 3).

Extract of strain RB51*leuB*/SOD/*wboA* was subjected to immunoblot analysis and screened against a rat monoclonal antibody Bru38 to *Brucella* O-side chain (32). Strain RB51*leuB*/SOD/*wboA* expressed O-side chain in similar profile as RB51/WboA did (29) and showed bands at around 35 – 50 kDa sizes (Fig. 4). Over-expression of WboA did not alter the rough phenotype of the strain as the colonies retained the stain when stained with crystal violet (data not shown). Also, in acriflavin solution, the strain

RB51*leuB*/SOD/*wboA* agglutinated suggesting that complementation of *wboA* did not alter its rough phenotype (data not shown).

Moreover, any over-expression of the antigens did not alter the biochemical characteristics of the vaccine strains. Tests for oxidase, catalase and urease activity showed that the recombinants retained the characteristics of the parent strain *B. abortus* strain RB51 (23). Growth in basic fuchsin and no growth in thionin containing media were consistent with parent strain characteristics (data not shown).

Attenuation characteristics unaltered in recombinant vaccine strains.

Over-expression of any of the three antigens, SOD, L7/L12 or O-side chain did not affect the attenuation characteristics of strain RB51*leuB*. At 1, 3 and 5 weeks post vaccination; the protective antigen over-expressing vaccine groups showed similar attenuation levels as that of the control strain RB51*leuB* as depicted by the similar bacterial loads in the spleen of the inoculated mice (Fig. 5).

Protection conferred by recombinant RB51*leuB* strains against *B. suis* challenge.

Mice vaccinated with strain RB51*leuB*/SOD/WboA afforded a significantly higher protection levels ($P < 0.0001$) against *B. suis* strain 1330 challenge when compared to mice vaccinated with saline (mean reduction in CFU/spleen = 3.54 log). Moreover, the protection afforded by strain RB51*leuB*/SOD/WboA was significantly different from other candidate strains; RB51*leuB*/SOD ($P = 0.0067$), RB51*leuB*/SOD/L7/L12 ($P = 0.0292$) and RB51*leuB* ($P = 0.0018$) as shown in Fig. 6. There was no significant difference in the protection levels afforded between the vaccine groups RB51*leuB*/SOD and RB51*leuB*/SOD/L7/L12 ($P = 0.9595$). However, there was a significant difference in the protection levels afforded by the vaccine groups

RB51*leuB*/SOD (P = 0.0287) and RB51*leuB*/SOD/L7/L12 (P = 0.0065) when compared to unvaccinated control (saline) group (mean reduction in CFU/spleen = 1.60-1.94 logs). The control vaccine strain RB51*leuB* with no antigens over-expressed did not protect mice significantly (P = 0.0954) relative to the control group vaccinated with saline.

Mice immune response to vaccine strains.

All mice vaccinated with any of the SOD over-expressing strain, produced SOD specific antibodies (Fig. 7A) when pooled sera from mice (n=5) were screened against purified SOD as antigen. The antibodies produced were biased towards IgG2a sub-isotype (Fig. 7A) and there was a significant difference in the IgG2a levels between different vaccine groups. Similar to previous observations (29) the IgG2a levels against SOD were lower in mice vaccinated with RB51*leuB*/SOD/WboA when compared to RB51*leuB*/SOD vaccinated mice. This trend towards IgG2a isotype suggests a predominantly Th1 type immune response against the over-expressed antigen SOD. Also, when purified *Brucella* LPS was used as antigen, the antibodies produced were biased towards IgG2a isotype (Fig. 7B).

Splenocytes of mice vaccinated with any of the vaccine strains produced higher amounts of IFN-γ upon stimulation with heat killed *Brucella*. However, there was no significant difference in the levels of IFN-γ produced between the following vaccine groups, RB51*leuB*, RB51*leuB*/SOD, RB51*leuB*/SOD/L7/L12, and RB51*leuB*/SOD/*wboA* (Fig. 8). The stimulated splenocytes from the saline group produced below detectable levels of IFN-γ when non-stimulated with *Brucella*, as similar to our observations that had *in vitro* stimulation (29). No detectable levels of IL-4 were present in the supernatant

of antigen stimulated splenocyte cultures prepared from any of the vaccine groups (data not shown).

DISCUSSION

In our earlier studies, over-expression of SOD in *B. abortus* strain RB51 enhanced protection against *B. abortus* infection (33); moreover cytoplasmic expression of O-side chain in strain RB51 not only enhanced protection in mice against *B. abortus* challenge but also cross-protected them against a virulent *B. melitensis* challenge (29). Complementation of strain RB51 with an intact *wboA* gene results in production of O-side chain, but the strain remained rough because of the cytoplasmic localization of the O-side chain and thereby retained the attenuation characteristics (32). These studies suggested that strain RB51 can be used as a platform to over-express *Brucella* derived protective antigens to produce a recombinant vaccine strain that could protect livestock not only against *B. abortus* but also against *B. melitensis* infection. In all the above-mentioned studies, we employed plasmids that were marked by antibiotic resistance cassettes. Regulatory bodies like the FDA discourage and strictly regulate the use of drug resistance genes in recombinant vaccines meant for field release (2, 6). To address this issue, we constructed and successfully employed an antibiotic resistance free vaccine platform using a leucine auxotroph of strain RB51 and a *leuB* complementing plasmid pNS4 for over-expression of protective antigens (19). In this study, we assessed the cross-protective efficacy of the antibiotic resistance free vaccine platform (*B. abortus* strain RB51*leuB* + pNS4) over-expressing protective antigens (SOD, L7/L12 and O-side chain) against a virulent *B. suis* 1330 challenge in mice in an effort to develop an effective vaccine against swine brucellosis.

Over-expression of Cu/Zn SOD in strain RB51*leuB* using pNS4 did not elicit enhanced cross-protection in mice against *B. suis* 1330 challenge (Fig. 6), a result similar to our earlier finding that used a *B. melitensis* challenge (29). This result suggests that SOD may not be a protective antigen either in *B. suis* or in *B. melitensis*, even though it has shown to elicit an enhanced protective immune response against *B. abortus* challenge in mice (33). The cytokine profile (IFN-γ and IL-4 levels) of strain RB51*leuB*/SOD vaccinated mice did not differ significantly (Fig. 8) from other candidate vaccines, particularly the strain RB51*leuB*/SOD/WboA that has elicited superior protection. The significant difference in the SOD specific IgG2a antibody response (Fig. 7A) did not translate into increased protection levels. This indicates that there maybe a difference in antigenic determinants between the three most pathogenic species of *Brucella* (*B. abortus*, *B. suis* and *B. melitensis*) that might determine their host range and severity of disease caused in respective host animals and in humans. A SOD mutant of *B. abortus* was found not to be attenuated when compared to wild type (9) although SOD is considered one of the antigens of *Brucella spp* (25). The attenuation levels of *B. suis* mutated for SOD expression is yet to be ascertained. Further studies comparing the immunogenicity of the three species when they are mutated for SOD expression would shed some light on role played by SOD in the pathogenesis of the three species particularly in *B. abortus*. The periplasmic location of the over-expressed Cu/Zn SOD in strain RB51/SOD (33), maybe eliciting a specific immune response and elicits protection against *B. abortus* challenge. Whereas, additional cell surface and periplasmic proteins may have a role to play in the pathogenesis of *B. suis* infection. Inside a host *Brucella* encounters dendritic cells (DC), an important component of innate immune system, that

recognizes pathogens (5). Cell surface proteins of *B. abortus* have shown to play a role in human DC maturation by specific TLR2 and TLR4 signaling (37). Therefore, from this study, it may be speculated that *B. suis* may have a different set of structural components in its cell wall and periplasmic space (other than SOD), that might activate a different signaling cascade during infection. DC maturation is necessary for sensitizing the immune system to induce a specific T-cell response and subsequently to clear the infection. It can be speculated that, additional cell wall and surface proteins other than SOD, especially the components of LPS may play a role in avoiding DC maturation and therefore help pathogenesis of *B. suis* in setting up an infection. These proteins, if identified could be used as a protective antigen with or without SOD in a vaccine construct to elicit a protective immune response against *B. suis* infection.

The ribosomal protein L7/L12 when over-expressed from a strong promoter (*trc*D) in strain RB51 protected mice against a *B. melitensis* strain 16M challenge (Ashraf Omar, Ph.D. Dissertation, 2007, Zagazig University, Egypt). In this study, L7/L12 was over-expressed using beta-lactamase (*bla*) promoter in the pNS4 platform in strain RB51*leuB*. The *bla* signal sequence was fused downstream of the *bla* promoter in the plasmid pNS4/SOD/L7/L12 to translocate the expressed L7/L12 protein to the periplasmic space (15). The expression of antigens in the periplasmic space is expected to enhance antigen presentation and elicit a better immune response (21). However, strain RB51*leuB*/SOD/L7/L12 did not seem to enhance protection significantly when compared to the strain RB51*leuB*/SOD, which expresses only SOD (Fig. 6). Moreover, there was no significant difference in the IFN-γ levels produced in the splenocytes of mice that were vaccinated with either the strain RB51*leuB*/SOD or RB51*leuB*/SOD/L7/L12 (Fig. 8)

when induced with heat killed *Brucella*. The antibody response against SOD shows that the strain co-expressing SOD and L7/L12 has a relatively higher IgG2a antibody levels (Fig. 7A) but that did not correlate with enhanced protection. Since SOD and L7/L12 has been used together successfully as a DNA vaccine (36), expression of one antigen should not interfere with other antigen presentation. One reason could be that in this case, the over-expression of SOD in its native periplasmic location is so strong that it minimized the amount of periplasmic L7/L12 presented to the immune system to elicit a specific immune response. Earlier it has been shown that L7/L12 expression induces a mixed Th1 and Th2 cellular immune response (11, 16). Since we did not have a study that used purified L7/L12 as antigen to screen for specific antibody response, we can speculate by indirect assessment of the type of immune response elicited by the strain RB51*leuB*/SOD/L7/L12 from the studies that used SOD and LPS as antigens. In those studies, the absence of a pronounced Th2 response characterized by relatively low IgG1 levels against the expressed antigens SOD and O-antigen (Fig. 7A and 7B) in mice vaccinated with strain RB51*leuB*/SOD/L7/L12 may explain the absence of enhanced protection in mice vaccinated with this strain. Alternatively, the strength of the *bla* promoter may not be sufficient enough to produce critical levels of L7/L12 to stimulate a significant protective immune response. Studies show that over-expression of L7/L12 from a stronger promoter, *trc*D, and localization of the protein in cytoplasm did enhance immune response against a *B. melitensis* challenge (Ashraf Omar, Ph.D. Dissertation, 2007, Zagazig University, Egypt). Cytoplasmic localization of over-expressed L7/L12 may be critical in antigen presentation in order to evoke a protective immune response against *B. suis* infection. Further studies addressing the promoter strength and

localization of over-expressed protein in cytoplasm should be pursued to elucidate whether L7/L12 is a protective antigen against a *B. suis* infection.

Complementation of the strain RB51*leuB* with gene *wboA* results in production of antigenic O-side chain, but still retains the rough phenotype and attenuation properties (Fig. 5). The rough phenotype was verified by staining the colonies with crystal violet in which they retained the stain suggesting that they are rough (data not shown). Also, in the acriflavin solution the bacteria agglutinated suggesting that complementation of *wboA* did not alter its rough phenotype (data not shown). Superior protection was afforded by the strain RB51*leuB*/SOD/WboA against virulent *B. suis* challenge when compared to the other three vaccine candidates (Fig. 6). The highly significant protection levels (3.5 log) compared to saline group against *B. suis* challenge were similar to our earlier findings that used a *B. melitensis* challenge (4 log) in mice using strain RB51/SOD/WboA (29). The cytokine IFN-γ levels produced by the splenocytes of mice vaccinated with RB51*leuB*/SOD/WboA were not significantly different from the levels induced by other candidate vaccine strains (Fig. 8). Although, found to be necessary for protection against brucellosis, increased or higher amounts of IFN-γ levels did not correlate to increased or higher protection levels (8). It could be speculated that other than IFN-γ, cytokines like TNF-α may have a role to play in the increased protection afforded by this strain. *Brucella* O-antigen has found to form a stable complex with MHC II molecules of antigen presenting cells (APC), which in turn induces co-stimulatory molecules for a specific T-cell immune response (7). So, in this case, formation of this complex maybe a reason for the enhanced protection observed in the strain RB51*leuB*/SOD/WboA vaccinated group. Purified *B. abortus* LPS (O-antigen is a component of LPS) by itself

were found not to induce DC maturation (37). Maturation of DCs is necessary for expression and secretion of cytokines and co-stimulatory molecules to prime a specific T-lymphocytes response (37). Smooth virulent *Brucella* prevent DC maturation to avoid immune response from host and thereby use those cells to replicate and help setup a chronic infection (5). In this case, the strain over-expressing O-antigen may present other cellular antigenic proteins along with O-antigen to the MHC II molecules of DCs and thereby facilitate DC maturation. This maturation of DCs sensitized with *Brucella* cell components along with O-antigen may prime a T-cell immune response that translates into enhanced protection against *B. suis*. Thus from the literature (17, 37), it can be speculated that structural components of *Brucella* cell wall like lipoproteins (37) and outer membrane proteins (17), were better presented to the adaptive immune system along with the cytoplasmic O-antigen in case of mice vaccinated with strain RB51*leuB*/SOD/WboA. When *B. abortus* 2308 LPS was used as antigen, higher antibody levels (Fig. 7B) observed in the pooled sera of mice vaccinated with RB51*leuB*/SOD/WboA compared to other vaccine groups. The other vaccine groups relative to saline control group elicited significant levels of antibodies against LPS despite the absence of WboA in them (Fig.7B). It could be speculated that the antibodies that were produced in these vaccine groups that were not complemented with WboA, may be against other components of LPS such as Lipid A, and not against O-polysaccharide. Alternatively, the LPS (received from Dr. Oliver He, University of Michigan) that were used to coat the plates might have carried other components of *Brucella* cell that resulted in the binding of corresponding antibodies. Based on the level of protection observed, it could also be inferred that just the induction of specific

antibodies to O-side chain may be essential for protection against *B. suis* in the definitive host, swine. This could only be proved by passive transfer of O-antigen specific antibodies into naïve mice and assessing the protection afforded against a virulent *B. suis* challenge.

Altogether, the significance of this study is that it shows that the leucine auxotroph of strain RB51 along with pNS4 not only over-expresses protective antigens but also can elicit similar protective immune responses as shown with conventional antibiotic resistance marked plasmid systems (4, 20, 30, 31, 34). The cytokine response (IFN-γ and IL-4 levels) also supports the data that strain RB51*leuB* mimics the type of immune response elicited by the parent strain RB51. The trend towards pronounced levels of IgG2a antibodies indicates that the immune response elicited is biased towards Th1 type, a case similar to parent strain RB51. The isolation of plasmid pNS4 (over-expressing protecting antigens) from *Brucella* recovered from splenocytes of mice 5 weeks post vaccination (data not shown) indicates that this leucine auxotroph system is as stable as the conventional over-expression systems. Thus, strain RB51*leuB* along with pNS4 can serve as an environmentally safe replacement for the conventional strain RB51 over-expression system that uses antibiotic resistance cassette. Additionally, it could be concluded that the recombinant vaccine strain RB51*leuB* over-expressing *wboA* on pNS4 maybe used as a trivalent vaccine against *B. abortus*, *B. melitensis* and *B. suis* infection in livestock as it elicits a significant protective immune response against all three strains under experimental conditions. Further experiments in natural hosts are needed to assess the efficacy of these candidate vaccines under field conditions and that will validate the already obtained mice experiments results.

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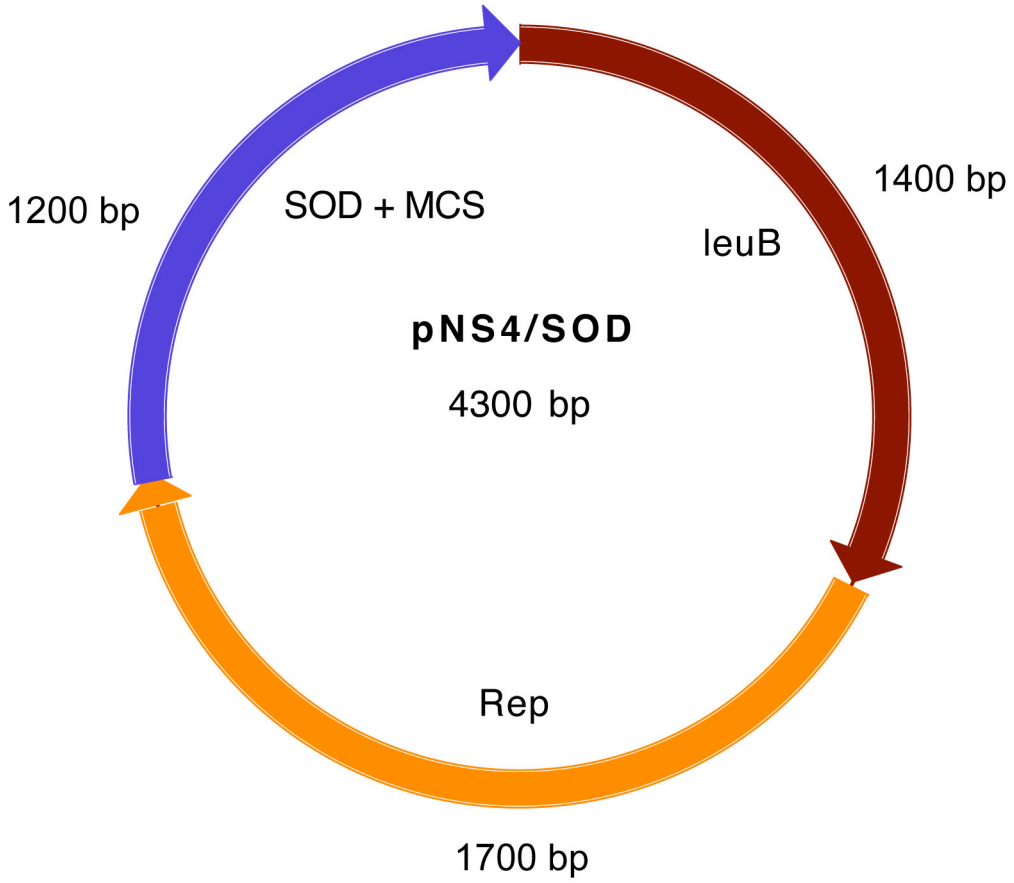


Fig. 1A Map of pNS4/SOD

MCS- Multiple cloning site

Rep – Origin of replication

leuB – intact *leuB* gene and its promoter region from *B. abortus*

SOD- Superoxide dismutase gene and its promoter region (*sodC)*

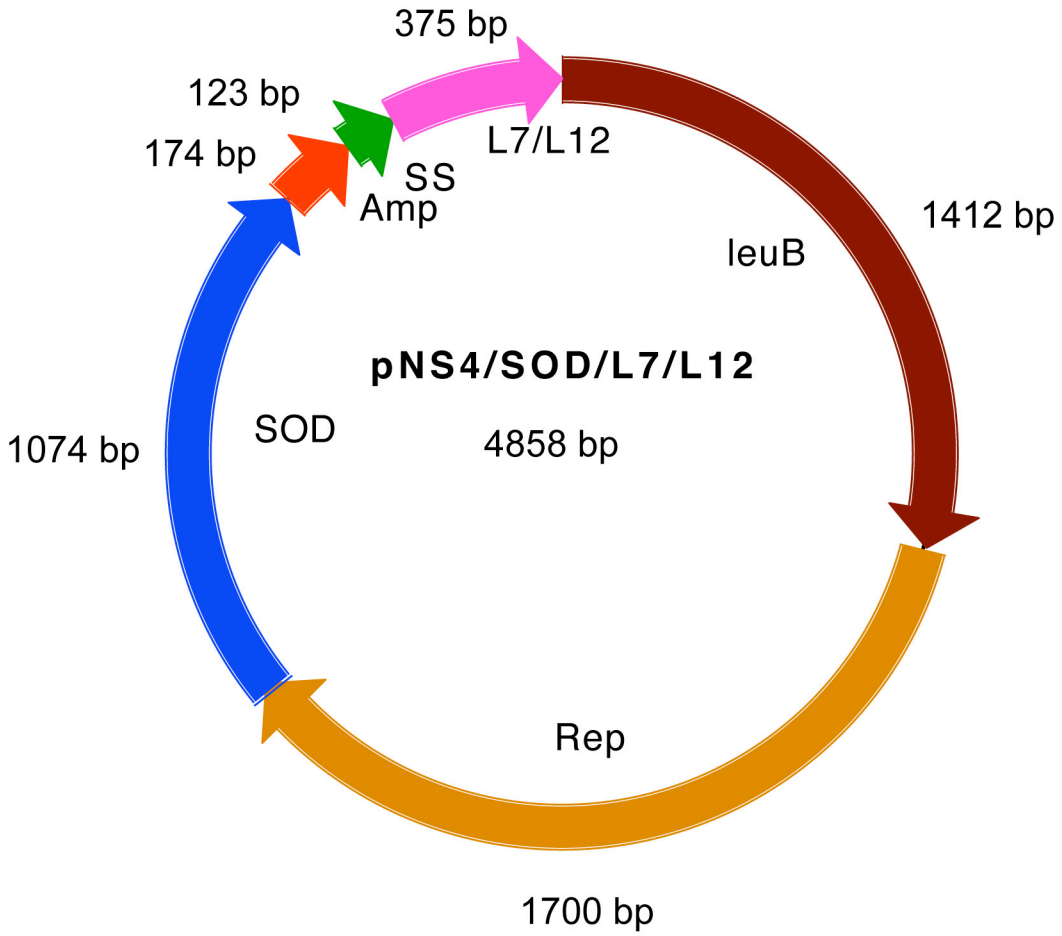


Fig. 1B Map of pNS4/SOD/L7/L12

Rep – Origin of replication

leuB – intact *leuB* gene and its promoter region from *B. abortus*

SOD- Superoxide dismutase gene and its promoter region (*sodC)*

Amp – *bla* promoter

SS- *bla* signal sequence

L7/L12- ribosomal protein *rplL*

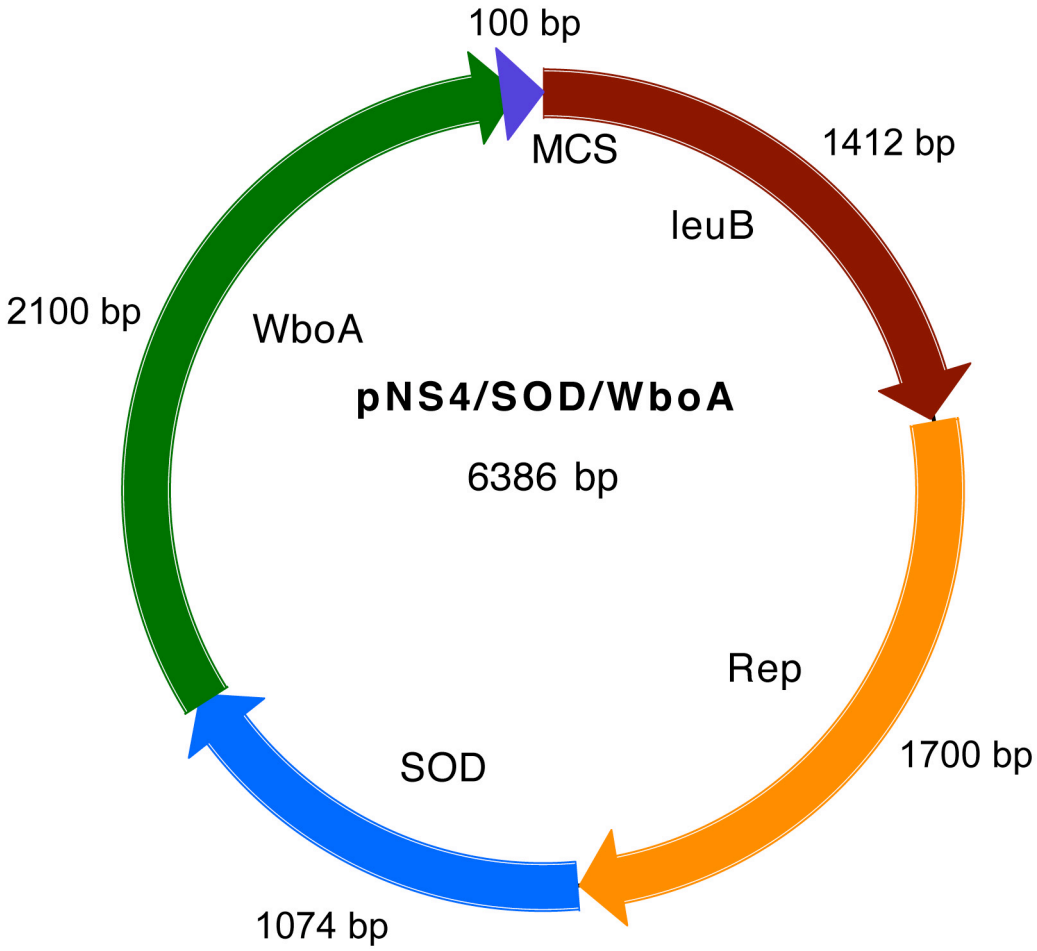


Fig. 1C Map of pNS4/SOD/WboA

Rep – Origin of replication

leuB – intact *leuB* gene and its promoter region of *B. abortus*

SOD- Superoxide dismutase gene and its promoter region (*sodC)*

WboA- glycosyl transferase gene and its promoter region *(wboA)*

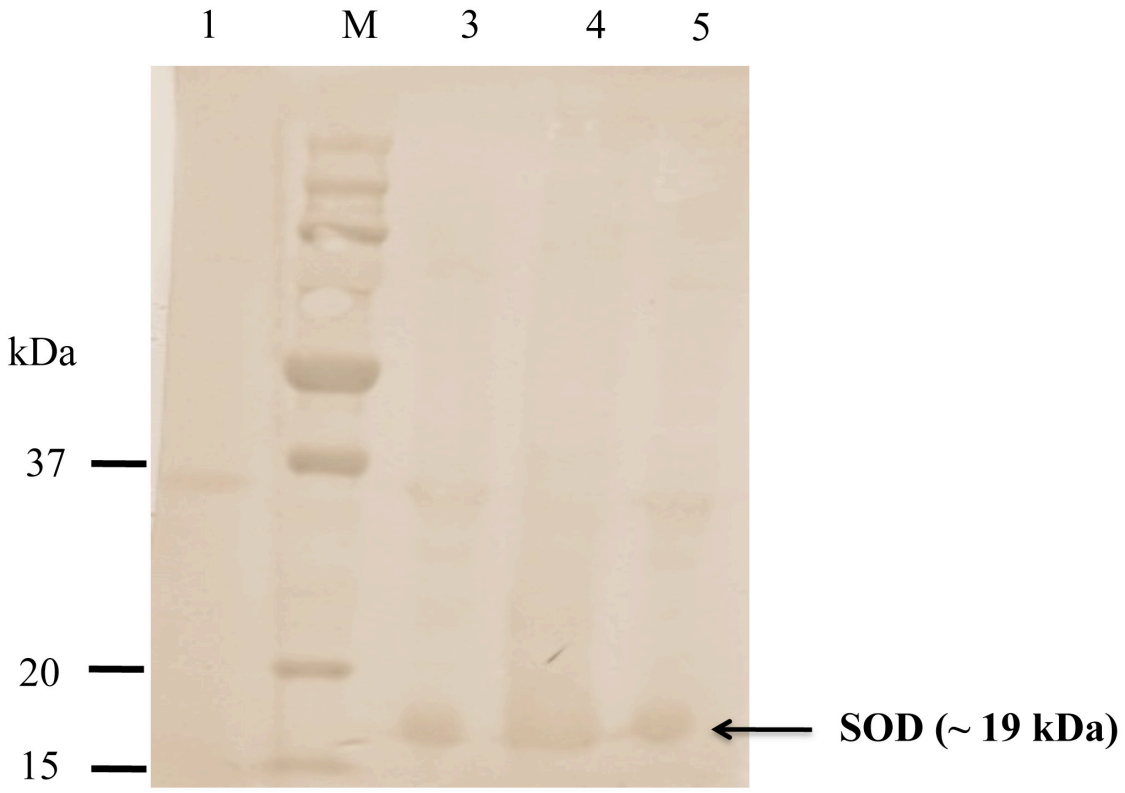


Fig. 2. Immunoblot for screening SOD expression: Lane - Sample; M-Standards, 1-RB51*leuB* (control), 3-RB51*leuB*/SOD, 4-RB51leuB/SOD/L7/L12, 5-RB51*leuB*/SOD/WboA. The bacterial antigens were separated on 10% Bis-Tris gel, transferred onto a nitrocellulose membrane and were screened using goat SOD antibodies (1:4000). SOD expression is seen at ~ 18 kDa region, and the band at ~ 37 kDa is most probably a dimer of SOD that has been observed in earlier studies (33).

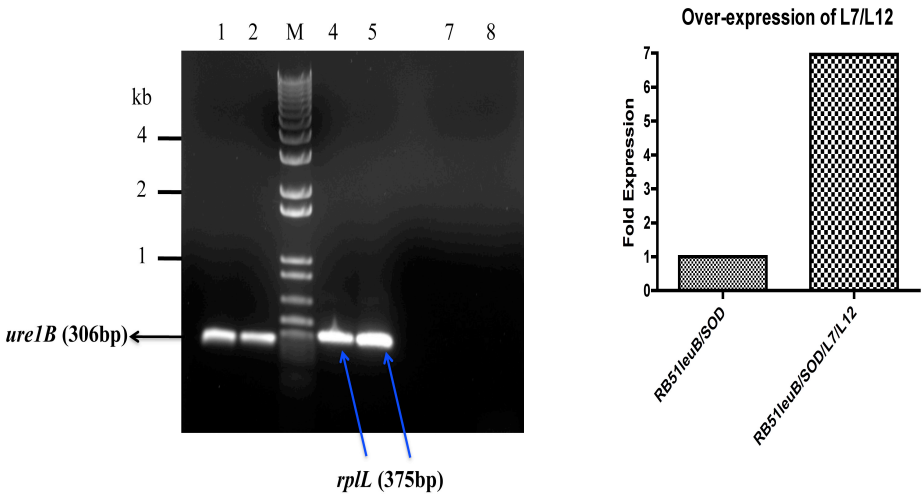


Fig. 3. Panel 1. Semi-quantification of PCR products of the genes *rplL* (L7/L12) and *ure1B* (beta subunit of urease 1 gene) amplified using cDNA as templates. Lane - cDNA template of the strain used (Gene (bp)); M-1kb plus DNA Ladder, 1- RB51*leuB*/SOD (*ure1B* (306bp)), 2- RB51*leuB*/SOD/L7/L12 (*ure1B* (306bp)), 4- RB51*leuB*/SOD (L7/L12 (375bp)), 5-RB51*leuB*/SOD/L7/L12 (L7/L12 (375bp)), 7- Negative control (*ure1B* primers) 8- Negative control (L7/L12 primers). PCR products electrophoresed on a 0.9% DNA agarose gel and the intensity of bands were analyzed on a densitometer. After normalization with house keeping gene *ure1B* expression, strain RB51*leuB*/SOD/L7/L12 (lane 5) was found to over-express the L7/L12 gene 27% more than strain RB51*leuB*/SOD (lane 4). **Panel 2**. **Quantification of L7/L12 over-expression using real time PCR.** The strain RB51*leuB*/SOD/L7/L12 with the plasmid-borne gene L7/L12 over-expresses 6 fold more than the strain RB51*leuB*/SOD that has only the chromosomal copy of gene *rplL*. The mRNA isolated from the strains was used as template for both the semi-quantification and real-time PCR analysis and the net over-expression was derived after normalizing with *ure-1B* gene expression.

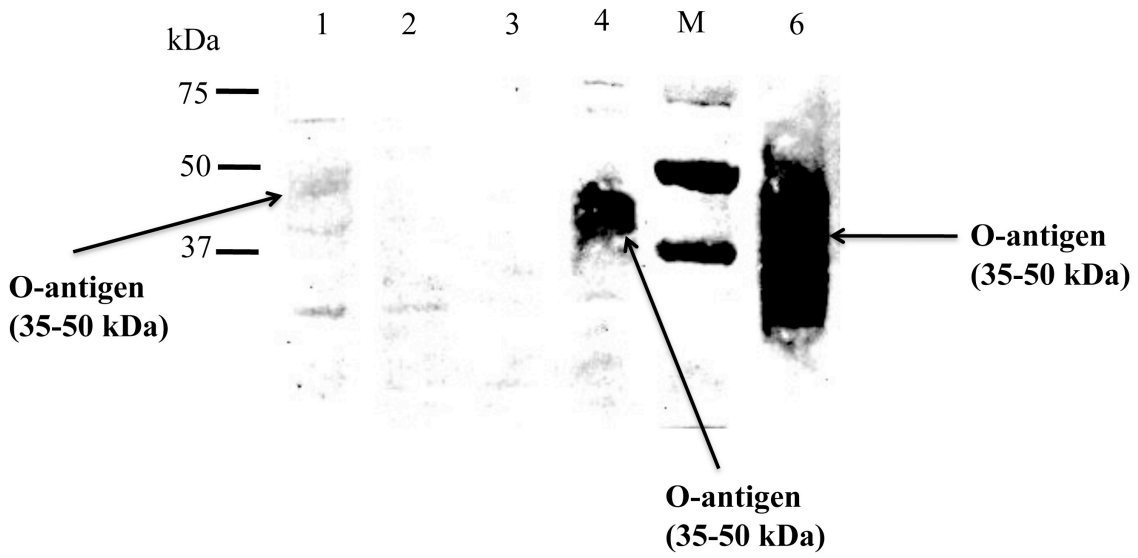


Fig. 4. Immunoblot for screening of O-antigen expression: Lane - Sample; M-Standards, 1- RB51*leuB*/SOD/WboA, 2- RB51*leuB* 3- RB51*leuB*/SOD, 4- RB51/WboA, 6- *B. abortus* S2308 (Positive control). The blot was screened using Bru38; a rat monoclonal antibody to *Brucella* O-side chain. The O-antigen can be seen as multiple bands with sizes varying from 35-50 kDa as the O-antigen is a homopolymer and varies in unit sizes upon separation. Strains RB51*leuB*/SOD/WboA, RB51*leuB*/SOD were grown on minimal medium (BMM) and strains RB51/WboA, RB51*leuB* and *B. abortus* S2308 were grown on enriched tryptic soy broth.

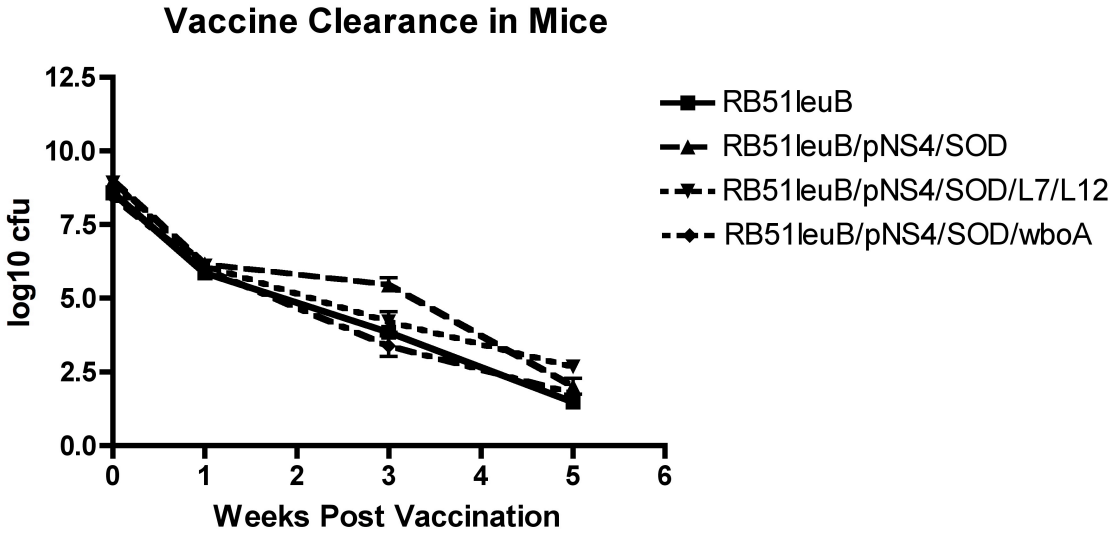


Fig. 5. Clearance rate of the recombinant vaccine strains in BALB/c mice. At 1, 3 and 5 weeks post vaccination, mice (n=5) vaccinated with different recombinant RB51*leu*B strains were euthanized and the bacterial loads in their spleen were determined by plating the serial dilutions of homogenized spleens. Over-expression of any of the protective antigens did not alter the attenuation characteristics (i.e. clearance) of the strains when compared to the control strain RB51*leuB*.

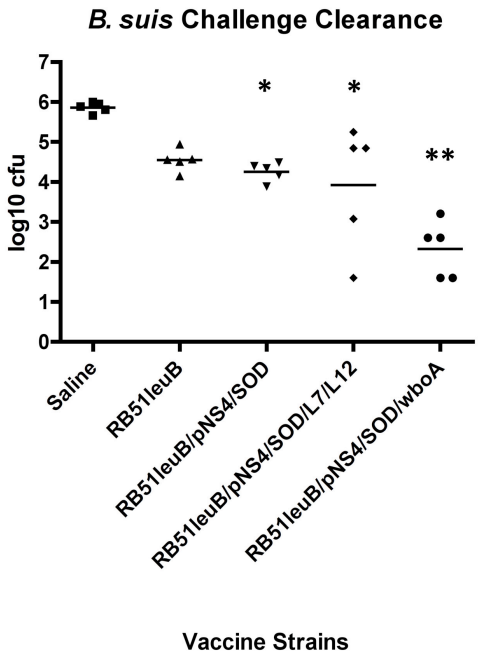
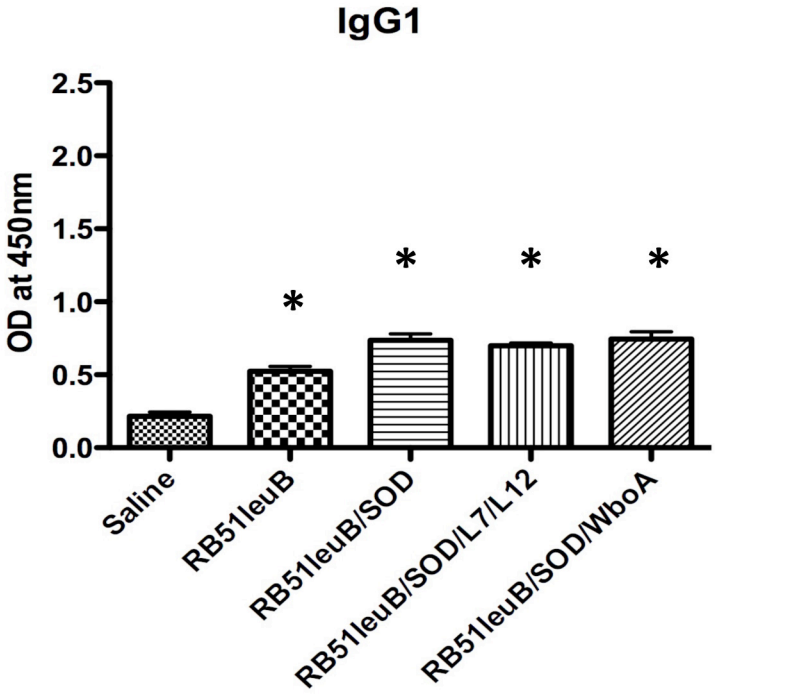


Fig. 6. **Clearance of the challenge strain *B. suis* 1330 in BALB/c mice vaccinated with different candidate vaccines.** Mice vaccinated with the recombinant RB51*leu*B strains were challenged with virulent strain *B. suis* 1330 at 6 weeks post vaccination. The efficacy of different candidate vaccines to clear the challenge strain was assessed by counting the number of CFU's in the spleens of the vaccinated mice two weeks post challenge. Strain RB51*leu*B/SOD/WboA vaccinated mice cleared the challenge strain very significantly ($P < 0.0001$). Strains RB51*leu*B/SOD ($P < 0.0287$) and RB51*leu*B/SOD/L7/L12 ($P < 0.0065$) also protected mice significantly ($P < 0.05$ is considered significant).


IgG1
OD at 450nm
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Saline
RB51leuB
RB51leuB/SOD
RB51leuB/SOD/L7/L12
RB51leuB/SOD/WboA

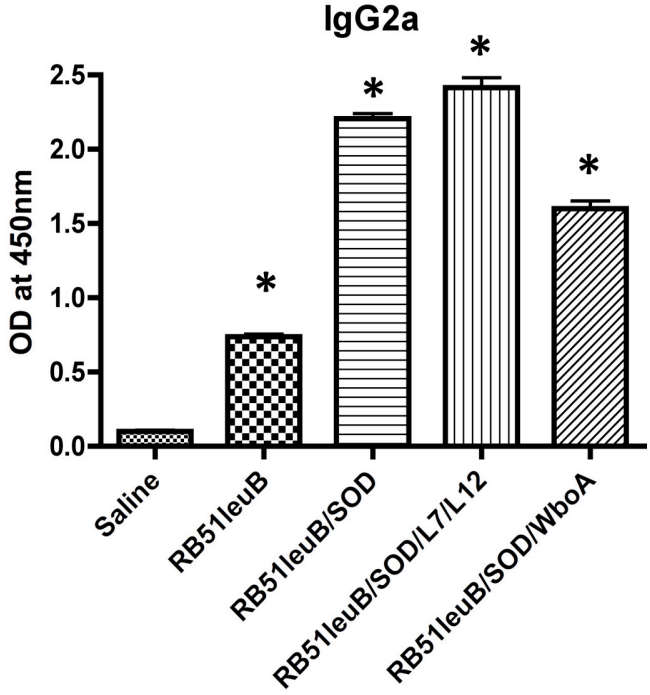
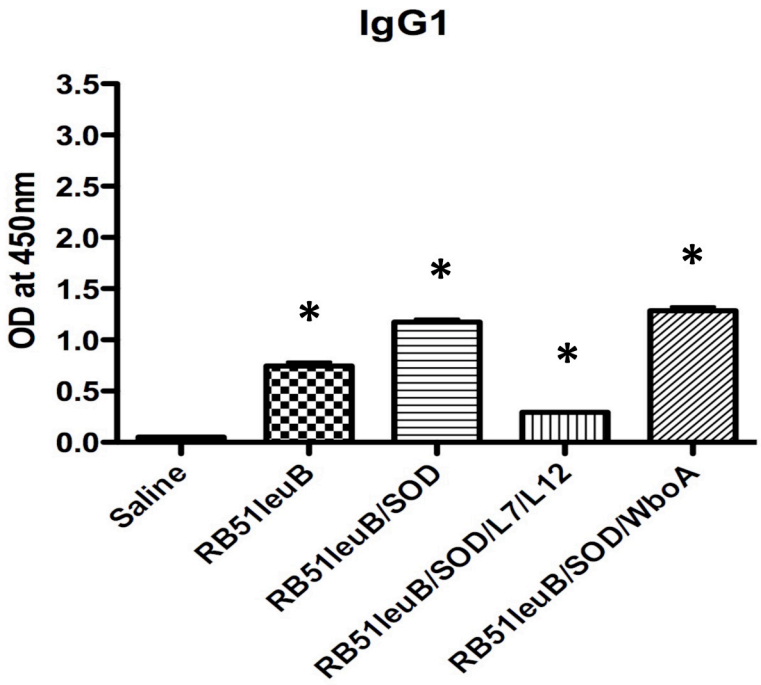

IgG2a
OD at 450nm
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Saline
RB51leuB
RB51leuB/SOD
RB51leuB/SOD/L7/L12
RB51leuB/SOD/WboA

Fig. 7A. Detection of Cu/Zn SOD specific antibodies in serum of vaccinated mice. Pooled sera from mice (n=5) collected 5 weeks post vaccination from different vaccine groups were diluted at 1:100 assayed for specific sub-isotype antibodies using purified SOD as antigen. All vaccinated groups produced significant levels of SOD specific IgG1 and IgG2a antibodies when compared to the saline control group. There was no significant difference in IgG1 levels produced between the different vaccine groups that are over-expressing any of the antigens. However, there was a significant difference in IgG2a levels produced between the different vaccine groups that are over-expressing any of the antigens. Also, there was a significant difference in both IgG1 and IgG2a levels between the control vaccine RB51*leuB* and the antigen over-expressing vaccine candidates. ($P < 0.05$ is considered significant)


IgG1
OD at 450nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Saline
RB51leuB
RB51leuB/SOD
RB51leuB/SOD/L7/L12
RB51leuB/SOD/WboA

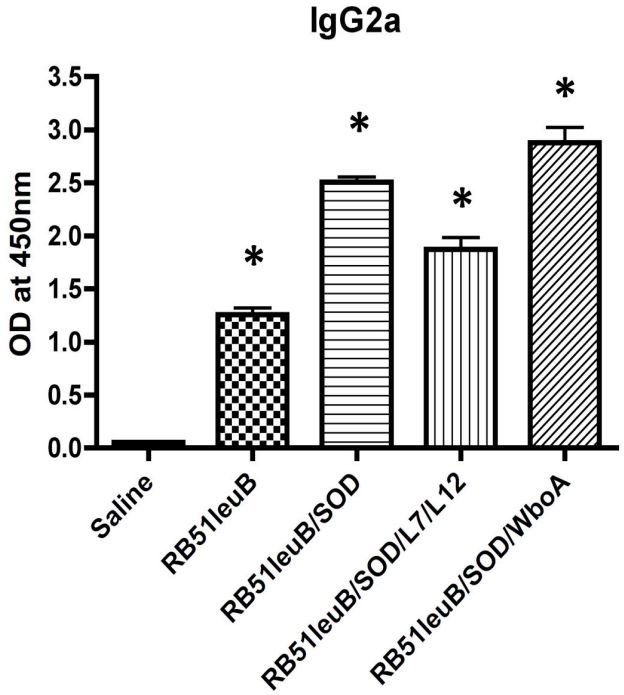

IgG2a
OD at 450nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Saline
RB51leuB
RB51leuB/SOD
RB51leuB/SOD/L7/L12
RB51leuB/SOD/WboA

Fig. 7B. Detection of *Brucella* LPS specific antibodies in serum of vaccinated mice. Pooled sera from mice (n=5) collected 5 weeks post vaccination from different vaccine groups were diluted at 1:100 and assayed for specific sub-isotype antibodies using purified *Brucella* LPS as antigen. All vaccinated groups produced significant levels of LPS specific IgG1 and IgG2a antibodies when compared to the saline control group. There was no significant difference in IgG1 and IgG2a levels produced between the vaccine groups RB51*leuB* and RB51*leuB*/L7/L12. Similarly, there was no significant difference in IgG1 and IgG2a levels produced between the vaccine groups RB51*leuB*/SOD and RB51*leuB*/SOD/WboA ($P < 0.05$ is considered significant)

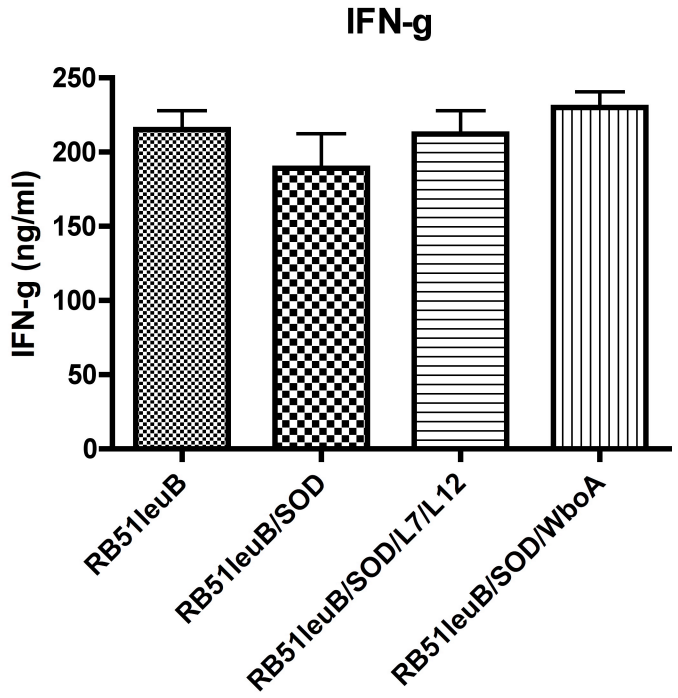


Fig. 8. Concentration of IFN-γ in the splenocyte culture supernatants upon stimulation by heat killed *Brucella*. Individual spleens from vaccinated mice (n=5) were harvested 7 weeks post vaccination and a single cell suspension of splenocytes was cultured in the presence of heat-killed *B. abortus* RB51 for 5 days. The concentrations of IFN-γ in the supernatant of the splenocyte culture (triplicates/mouse) were quantified by indirect sandwich ELISA. No significant difference was found in the levels produced between the four vaccine groups. The non-stimulated saline control group produced levels of IFN-gamma below detection. The lowest detection limit was set at 156 pg/ml. ($P < 0.05$ was considered significant).

Chapter Four

Evaluation of the ability of strain RB51*leuB* to express native and *Brucella* optimized influenza (heterologous) antigens

Abstract

Swine influenza has the potential to cause a global pandemic in humans that could result in countless mortality and enormous economic burden. An effective and safe vaccine that could protect swine from all the influenza A virus strains would be one of the strategies to prevent an influenza pandemic in humans. Swine can be infected by influenza A viruses that infect swine, poultry and humans. Thus, swine as a "mixing vessel" provide the environment for reshuffling of RNA genome segments among these three influenza strains, which could result in the emergence of a highly pathogenic pandemic strain. An immune response that is cross protective against all influenza A viruses would minimize the chance of re-assortment of genetic segments in swine. Since *Brucella abortus* strain RB51*leuB* possesses the ability to deliver antigens at the intracellular level, a long term T-cell immune response is expected for an influenza antigen delivered via this platform. The protective antigenic regions of three influenza antigens, hemagglutinin, nucleoprotein and matrix protein were cloned via a plasmid into the leucine auxotroph of strain RB51. No expression of the native viral genes was detected on an immunoblot of extracts from the transformed strain RB51. However a reverse transcriptase PCR analysis revealed that viral gene specific mRNAs for all three antigens were produced that suggested some type of translational problem. Following

optimization of the viral genes for *Brucella* preferred codon usage, the viral genes were cloned onto the RB51*leuB* platform. Again, no expression of the viral genes was detected in immunoblots; however RT-PCR revealed that specific viral mRNA was transcribed. As influenza genes have been expressed in *Escherichia coli*, it will be necessary to further refine their expression for the RB51*leuB* platform.

Introduction:

Swine are considered an important "mixing vessel" for avian and human influenza viruses as they possess receptors for both avian and human strains and thereby can be infected by both (34). Avian influenza strains bind to Neu5Acα2-3Gal receptors of the enteric tract of birds and human influenza strains bind to Neu5Acα2-6Gal receptors of the human respiratory tract (17). Since swine respiratory tract possess both the receptors they can get infected with avian and human type of strains and as well as swine influenza strains (12). Re-assortment of genomic RNA segments among co-infecting swine, avian and human influenza viruses may result in a highly pathogenic strain that could cause a global pandemic (2). The recent 2009 pandemic of swine influenza strain A/H1N1 is a classic example of this re-assortment phenomenon as it carries genetic segments from influenza A strains that infect all three hosts (13). The global pandemic caused by this novel virus in humans accounted for 300,000 infections and 3917 deaths across 191 countries as of 09/25/2009 (http://www.who.int/csr/don/2009_09_25/en/index.html). The extent of influenza spread led the World Health Organization (WHO) to raise the global pandemic alert to the highest level "six", as the virus has exhibited unseen combinations of RNA gene segments (13). Though the virus is

sensitive to some available antiviral drugs (Oseltamivir and Zanamivir), there have also been some drug resistant strains identified (4). Apart from infecting humans and swine, the strain H1N1 was found to be infecting cat and ferret population, a United States Department of Agriculture (USDA) report states dated November 4, 2009 (http://www.aphis.usda.gov/newsroom/hot_issues/h1n1/). A recent study conducted by the Agricultural Research Services (ARS) states that the pre-existing immunity in the United States swine herds couldn't protect the population from contracting the 2009 pandemic strain (http://www.ars.usda.gov/2009H1N1/). The study also suggests that the currently used swine influenza vaccines may not protect swine herds from the new pandemic H1N1 strain infection. Together these observations reflect the need for an effective containment strategy to prevent outbreaks caused by swine origin influenza viruses.

Vaccination has proven to be the most effective tool in prevention, control and eradication of the most destructive pandemic viral diseases e.g. small pox and polio. Although flu vaccine research has been actively pursued (8), a cross protective universal vaccine against a pandemic swine flu is still an elusive goal. Even after identifying a seed virus for the pandemic strain, producing and dispensing the attenuated virus vaccine on a global scale would be a phenomenal challenge both in terms of cost and infrastructure (14). According to a WHO report, in United States alone, influenza related epidemics (not a pandemic) cost $ 71-167 billion of economic burden during 2002 – 2003 (http://www.who.int/mediacentre/factsheets/2003/fs211/en/). All the above-mentioned issues warrant a broad range vaccine that would prevent re-assortment among the avian and human virus types in a swine host thereby minimizing the chance of a pandemic in

humans. The ideal vaccine for swine should induce immunity against the avian and human flu strains while also eliciting a protective immune response against the swine influenza strain. Multivalent or polyvalent vaccines could protect swine against infection from different strains of influenza virus. Vaccine vectors like adenoviruses have been used to deliver influenza antigens from more than one strain and thereby eliciting immune responses in swine against multiple influenza strains (37). Some bacterial vectors like *Salmonella* have been investigated to deliver immunogenic influenza proteins like hemagglutinin (HA) on plasmid DNA as a vaccine development strategy and has proven to be successful in priming the immune response (26).

Hemagglutinin (HA) and neuraminidase (NA) are the two major surface glycoproteins that determine the host range and antigenicity of the influenza viral strains (18). The globular head of the HA protein helps the influenza virus attach to the sialic acid containing oligosaccharide receptors on the surface of the host target cells (18). Antibodies against HA that are neutralizing prevent attachment of virus to the host cell (35). Among the epitopes of HA, an antibody response (humoral) to the globular head domain has shown to be more protective as it prevents viral attachment to host cell (18). Because of its high immunogenicity, the globular head of HA has been used as a vaccine under experimental conditions either as naked DNA, as a peptide fragment or fused with antigenic flagellin (18, 32). Most of the experiments in which a part of HA or the complete HA (1701 bp) was used as a antigen, did not elicit a desired response, i.e., a protective immune response from both B-cell and T-cell arms of the immune system (18, 19). Some of the failures were due to poor presentation of antigen to the immune cells, because of the conformational restrictions posed by a trimeric form of native HA

molecule (18). The trimeric form of whole HA molecule may not have exposed the receptor binding site to the immune system well enough to elicit a specific immune response. As an alternate, when only the globular head domain region containing the receptor binding pocket was used as a DNA vaccine, specific immune response was elicited and conferred partial protection against a viral challenge in mice (18). Using an effective delivery system that would overcome the previously experienced antigen presentation issues and could potentiate the host protective immune response against the globular head domain (271^{th} – 783^{th} bp) of HA is the step forward towards influenza vaccine development.

Nucleoprotein (NP) is the structural protein that encapsulates the viral RNA and acts as a switching factor to determine if the viral RNA is transcribed into mRNA for translation into protein or cRNA for genome replication and therefore is a "key adaptor between the virus and the host cell" (24). As an internal viral protein that has a highly conserved sequence, NP can be used as a prophylactic and therapeutic vaccine candidate as it would restrict viral replication by inducing T-cell mediated immunity (24). An immune response against the least variable NP region was found to be cross protective (9). Localization of the whole NP in the host nucleus when administered as a naked DNA compromised the immunogenicity of the vaccine construct because of poor antigen presentation (3). Improved but not desirable immune responses were observed when the whole NP was fused to either homologous or heterologous secretory proteins (which translocated the antigen outside nucleus) through better antigen presentation (20). Over-expression of only the most antigenic region of the NP and improved antigen delivery and presentation to immune cells would address the shortcomings experienced in

previous experiments. The first 540 bp of the whole NP (1496 bp) from the N-terminal region has the RNA binding groove and other antigenic epitopes that are potential vaccine target regions (24). A delivery system that can present this antigenic segment of NP to the innate immune system would trigger a protective and long term T-cell response.

In a cross-protective universal flu vaccine development effort, eliciting immune response against the conserved envelope protein M2 (97 amino acids) is one approach (11). A trans-membrane viral ion channel, the M2 protein tetramer is essential for un-coating of viral particles both for entry and egress from host cells (22). The nearly invariable, 23 amino acid residue, extracellular domain of M2 protein denoted as M2e, is being explored as a universal vaccine candidate (5, 10, 27). A synthetic M2e peptide used as a vaccine, protected mice against a lethal influenza challenge by eliciting both IgG antibody mediated and specific T-cell mediated immune response (38). The M2e peptide was also fused with other immunogenic proteins as conjugate vaccines (6, 11). Since M2e is a small peptide of only 23 amino acids in length, fusion with other proteins or fusing multiple copies of M2e peptides will augment immune responses (39). Intracellular delivery of a fused oligomer of M2e is expected to elicit a stronger B-cell and T-cell mediated immunity as opposed to a single copy of M2e.

Any of the discussed antigenic epitopes when presented through a safe and effective delivery system are expected to elicit cross-protective B-cell and T-cell immune responses. As discussed in the previous chapters, the vaccine strain *Brucella abortus* RB51*leuB*, and the antibiotic resistance free plasmid pNS4 have the ability to express and deliver antigens at an intracellular level. Expression of these flu antigens in a *Brucella*

platform and transporting the antigens to the periplasmic space should allow for an effective antigen presentation and therefore elicit an improved immune response. In this chapter, the ability of the strain RB51*leuB* to express the heterologous flu antigens will be described.

Materials and methods

Bacterial strains, media and growth conditions.

The *Escherichia coli* strain HB101 used was from our culture collection. Z-competent *E. coli* HB101 (Zymo Research, Orange, CA) were prepared as per the manufacturer's instructions and used for generating plasmid constructs. The *B. abortus* strain RB51*leuB* from our culture collection was made competent as described earlier (23, 29). *Brucella* minimal medium (BMM) was prepared as described by Plommet (28); all incubations were done at 37^{o}C in the presence of 5% CO_2. Transformation of *Brucella* was done as described previously (23) and the transformants were selected on BMM plates. Standard laboratory procedures recommended by Centers for Disease Control and Prevention were followed when culturing or handling live *Brucella*; all such work were performed in a Bio-safety level 3 facility of the Virginia-Maryland Regional College of Veterinary Medicine.

Construction of RB51*leuB* strains containing influenza genes: strains RB51*leuB*/SOD/HA, RB51*leuB*/SOD/NP, RB51*leuB*/SOD/bruHA, RB51*leuB*/SOD/bruNP, RB51*leuB*/SOD/bruM2e4 and RB51leuB/SOD/bruM2e4::HA

The plasmid pNS4/SOD/L7/L12 (from our culture collection) was linearized using restriction enzymes *BamHI* and *XbaI,* which deleted the *rplL* (L7/L12) gene. The gene HA was amplified from influenza A/PR/8 (H1N1) as a 516 bp fragment using the primer pair (Forward 5`- GGG**GGATCC**AGATCATGGTCCTACAUUGUAGAA-3` and reverse 5`-GGG**TCTAGACTCGAG**ATTACCATTTGCCTCAAATATTATTGTG -3') and was made to have sticky ends using restriction enzymes *BamHI* and *XbaI.* The HA gene was ligated with the similarly cut plasmid pNS4/SOD to produce pNS4/SOD/HA (Fig. 1A). The gene NP was amplified from influenza A/WSN/33 (H1N1) as a 540bp fragment using the primer pair (Forward 5`-GGG**AGATCT**ATGGCGACCAAAGGCACCAAACGA-3` and reverse 5`GGG**ACTAGTCTCGAG**ATTACACCTGCGGCCCCAGACCTCCTAGGGA-3`) and the ends were made sticky by *BglII* and *SpeI* restriction enzymes. The NP fragment was ligated into the linearized pNS4/SOD and transformed into Z-competent *E. coli* HB101 and the transformants were selected on leucine deficient minimal media plates to produce pNS4/SOD/NP (Fig. 1B). The protective antigens HA and NP were cloned such that they are driven by the *bla* promoter and would be translocated to the periplasmic space of *Brucella* by the beta-lactamase signal sequence for better presentation to the immune system (31). The *bla* promoter region includes the 174 bp upstream region of the gene and 105 bp of the actual *bla* gene containing the signal sequence to aid the translocation of flu antigen cloned downstream.

The sequences of HA, NP, M2e4::HA were optimized for *Brucella* codon usage by the GenScript Corporation, NJ and termed as brHA, brNP and brM2e4::HA. The construct brM2e4::HA has the ectodomain region of ion channel protein M2. The

ectodomain M2e (23 amino acids) used in this construct was an oligomer of M2e consisting of four different sequences from four different strain sources. The four repeats consists of M2e amino acid sequences from i) consensus sequence ii) H5 A/Hong Kong/156/97 iii) A/FM/1/47 and iv) A/California/14/2009. In the brM2e4::HA construct, the transmembrane domain and cytoplasmic tail domain of the viral HA gene (252 bp) was fused downstream to the four repeats of M2e sequence. All the genes were supplied on the commercially available plasmid construct pUC57. The gene fragments brHA and brM2e4::HA were excised from pUC57 using *BamHI* and *XbaI* sites and cloned onto the similarly linearized pNS4/SOD to produce pNS4/SOD/brHA and pNS4/SOD/brM2e4::HA (Fig. 1C). The brNP gene was excised using *BglII* and *SpeI* sites from pUC57 and then cloned on to the *BamHI* and *XbaI* linearized pNS4/SOD to produce pNS4/SOD/brNP. The brM2e oligomer (four M2e segments fused together consecutively) alone was amplified as a 276 bp fragment using the primer pair (Forward 5`- **GGATCC**TCGCTGCTTACCGAAGTGGAAACGCCGATCCGC -3` and reverse 5`-**TCTAGACTCGAG**ATTAGTCGGACGAATCCGAGCAGCGGCA -3') and cloned onto the linearized pNS4/SOD/AmpSS using *BamHI* and *XbaI* sites to produce pNS4/SOD/brM2e4 (Fig. 1D). All the plasmid constructs were transformed onto strain RB51*leuB* and the transformants were selected on BMM plates. The sequences of the codon-optimized genes compared to the native genes are presented in Fig. 2A, 2B and 2C. The expression segment of the plasmid containing the *bla* promoter, *bla* signal sequence and the optimized influenza antigen's genes and the expected amino acid sequences are provided in Fig. 6A, 6B, 6C and 6D.

Immunoblot.

The cell extracts of candidate vaccine strains were prepared using 10mM Tris-HCl buffer, as previously described (36). The extracts were electrophoresed in a 10% Bis-Tris gel (Invitrogen Corporation, Carlsbad, CA) and transferred onto a 0.45 μm nitrocellulose membrane (Trans-Blot, BIO-RAD Laboratories, Hercules, CA). Goat anti-RNP (Ribonucleoprotein) serum (1:3000) or mouse antibody to HA (1:3000) (Biodesign International, ME) or Mouse monoclonal M2e antibodies (clone 14C2, 1:3000) (ABCAM, MA) were used as primary antibodies. Peroxidase conjugated anti-goat or anti-mouse antibodies (ICN Pharmaceuticals Inc.) were used at 1:2000 as secondary antibodies. CN/DAB with stable peroxidase substrate (PIERCE, Rockford, IL) was used to detect the secondary antibody binding as per manufacturer's instructions. The goat anti-RNP antibodies were received from the NIH Influenza Research Reference and Reagent Program (National Institute of Allergy and Infectious Diseases, Maryland).

Reverse-Transcriptase PCR (RT-PCR)

Brucella RNA was isolated from bacterial cultures (~ 10^7 cfu) grown on BMM plates. The bacteria were harvested from plates by scrapping and incubated in RNA protect (Qiagen) at room temperature for 5 minutes to stabilize the RNA before being harvested by centrifugation in a microfuge at 5000 rpm (2000 x G) for 3 minutes. The pelleted cells were resuspended in 1 ml of TRIzol (Invitogen) and transferred into a tube containing micronium beads (FastRNA Pro Blue Kit, Q-BIOgene). *Brucella* was lysed on a Mini-bead beater 3110BX (Biospec products, OK) and then 0.1 ml of 1-bromo-3-chloropropane (Sigma) was added and mixed well followed by incubation for 2-3 minutes at room temperature. The colorless upper phase containing RNA was carefully transferred into a fresh tube after centrifugation at 12,000 x g for 15 minutes at 4°C. An

equal volume of 70% ethanol was added to the fresh tube containing RNA and mixed well to avoid any precipitation. The whole mixture was later transferred to the column of the PureLink kit (Invitrogen) and final washing and elution steps were followed as per manufacturer's instructions. The DNAse (Qiagen) treated RNA elution was used as a template to synthesize cDNA using the iscript cDNA synthesis kit (Bio-Rad laboratories) following the manufacturer's instructions in which random hexamers were used as primers.

Results and Discussion:

The strain RB51*leuB* transformed with either pNS4/SOD/HA or pNS4/SOD/NP showed no expression of either HA or NP on an immunoblot. The immunoblot was repeated twice to confirm the absence of expression of viral antigens. The positive control (virus infected cell lysate) for HA and NP expression reacted with the antiserum and revealed that the antibodies used are specific for the expressed influenza antigens. Verification for the presence of plasmids containing the influenza genes was done by both plasmid isolation and PCR amplification using influenza gene specific primers (Fig. 3). Since no expression was detected at the protein level for the influenza proteins, RT-PCR was done to detect transcription of influenza genes at the RNA level. When HA and NP gene specific primers were used on the cDNA template (reverse transcribed from RNA of the RB51*leu*B vaccine strains), amplification of corresponding gene sizes suggests mRNA had been produced but not translated into protein. One possibility is that the production of unstable mRNA of the transcribed influenza genes inside *Brucella* resulted in unavailability of critical amounts of mRNA for translation into protein. Otherwise, because of a highly skewed codon usage for influenza genes relative to the

host, *Brucella* codon usage could have affected the expression of viral antigens in vaccine strain RB51*leuB*.

Since *B. abortus* strain RB51 has been shown to express antigen (PA gene) from *Bacillus anthracis* (1), it was decided to optimize the codons of the influenza genes to suit the more preferred codon usage of the *Brucella* tRNA. Also, it had been shown that modifying the coding sequence and not the individual amino acid will increase the expression of a foreign protein in a bacterial platform (21).

The gene sequences of HA, NP and an oligomer of the ectodomain of matrix protein designated as M2e4 were optimized (GenScript Corporation, Piscataway, NJ). An oligomer of M2e was optimized because oligomeric conformation of four copies of M2e is expected to elicit a better immune response as shown previously (5). The optimized oM2e4 had four consecutive copies of the 23 amino acids each (276 bp) followed by a 252 bp of HA. The four consecutive copies of M2e are derived from three different strains of influenza virus (H5 A/Hong Kong/156/97, A/FM/1/47, A/California/14/2009) and the fourth copy is the consensus sequence of M2e (SLLTEVETPIRNEWGCRCNDSSD). Four different copies of M2e were used in order to protect against a broad range of influenza viruses. Four different constructs of RB51*leuB* were made using the complementing pNS4 as a base plasmid to over-express the optimized HA, NP, M2e4::HA and M2e4 genes. The 23 amino acid long brM2e4 gene segment alone was PCR amplified from the brM2e4::HA construct and used as a different construct to elucidate the difference in expression, if any, when compared with the construct that has brM2e4::HA. Immunoblots of the optimized influenza proteins showed no positive bands when specific antibodies were used (Fig. 4). Another

immunoblot loaded similarly was developed with the ECL detection kit (PIERCE, IL) which has higher sensitivity than the CN-DAB substrate kit, also showed no positive bands, thus suggesting that the absence of expression was not due to poor sensitivity of detection kits. In another separate immunoblot, again, no expression of influenza proteins was detected when Guanidine hydrochloride (GuHCl) at 6M concentrations was used to prepare bacterial lysates. In this case, 6M GuHCl was used to prepare bacterial lysates to dissolve insoluble proteins, if any, which were present inside bacteria as inclusion bodies. So, this suggested that influenza proteins were not present as insoluble products inside *Brucella*. Then, mRNA from all the four RB51*leuB* vaccine constructs over-expressing optimized flu genes (brHA, brNP, brM2e4 and brM2e4::HA) were isolated and reverse transcribed into cDNA. The cDNA was used as a template to amplify brHA, brNP and brM2e4 gene segments for verification of mRNA transcription using specific primers. The amplified gene fragments were of expected sizes (Fig. 5) indicating that again, mRNA was transcribed but did not get translated into protein. Suitable negative controls (The mRNA isolated from the vaccine strains was used directly as template for PCR amplification of specific influenza genes) were used.

Since the influenza proteins were not expressed in vaccine strain RB51*leu*B even after optimizing the codon usage to suit the *Brucella* tRNA preference, alternate strategies must be pursued to develop a *Brucella* expressed vaccine against swine influenza. One of the strategies might be to fuse the influenza antigens to another bacterial antigen, preferably a *Brucella* antigen, on the plasmid construct. Fusion of influenza antigens like HA with homologous antigenic proteins from bacteria e.g. HSP60 and Esat-6 from *Mycobacterium tuberculosis*, did enhance immunogenicity and shown to

elicit an improved humoral immune response against influenza challenge (7, 11, 25, 32). For example, it would be useful if any of the three optimized influenza antigens (brHA, brNP, brM2e4, brM2e4::HA) were fused to the *Brucella* Cu-Zn superoxide dismutase (*sodC*) protein to determine if the fused genes were transcribed, translated and translocated to the periplasm of *Brucella* strain RB51*leuB*. Since the Cu-Zn SOD is periplasmic (33), the fused influenza antigens are expected to be expressed and transported to the periplasmic space and may have a better antigen presentation. The antigenicity of SOD may also potentiate the immunogenicity of the fused protein as it has been shown earlier in our laboratory for anthrax PA expression in strain RB51 (S. Poff, Ph.D. Dissertation, Va Tech).

Another approach would be to use *Brucella* as an intracellular delivery vehicle for a plasmid based DNA vaccine. This approach requires that a transformed *Brucella* strain will not survive inside the phagosome of phagocytic cells (e.g. macrophages), but will release the plasmid DNA during lysis. A large percentage of the *Brucella* strains including strain RB51 that infect macrophages are lysed and thus fulfill this requirement (15). Influenza antigens fused to heterologous antigenic proteins as DNA vaccines have shown to be immunogenic (25). Multivalent DNA vaccines using eukaryotic promoters to express influenza antigens have shown to be protective in mice (30). Once intracellular, the recombinant *Brucella* would lyse and thereby release the plasmid DNA, which would then use host cell machinery to facilitate expression of the gene of interest. This should elicit a T-cell immune response to the plasmid-expressed influenza antigens. One of the advantages of using a *Brucella* based delivery system is that the Th-17 arm of the T-cell immune response would also be sensitized (16). The Th-17 arm of immune

response has been shown to play a role in protection against infectious diseases, including brucellosis, among aged mice (16). Using a *Brucella* based vaccine would thereby address the issue of eliciting a protective immune response against influenza infections among older animals. Thus, developing a swine influenza vaccine on a *Brucella* platform provides unique advantages as discussed above and should be pursued with alternate strategies.

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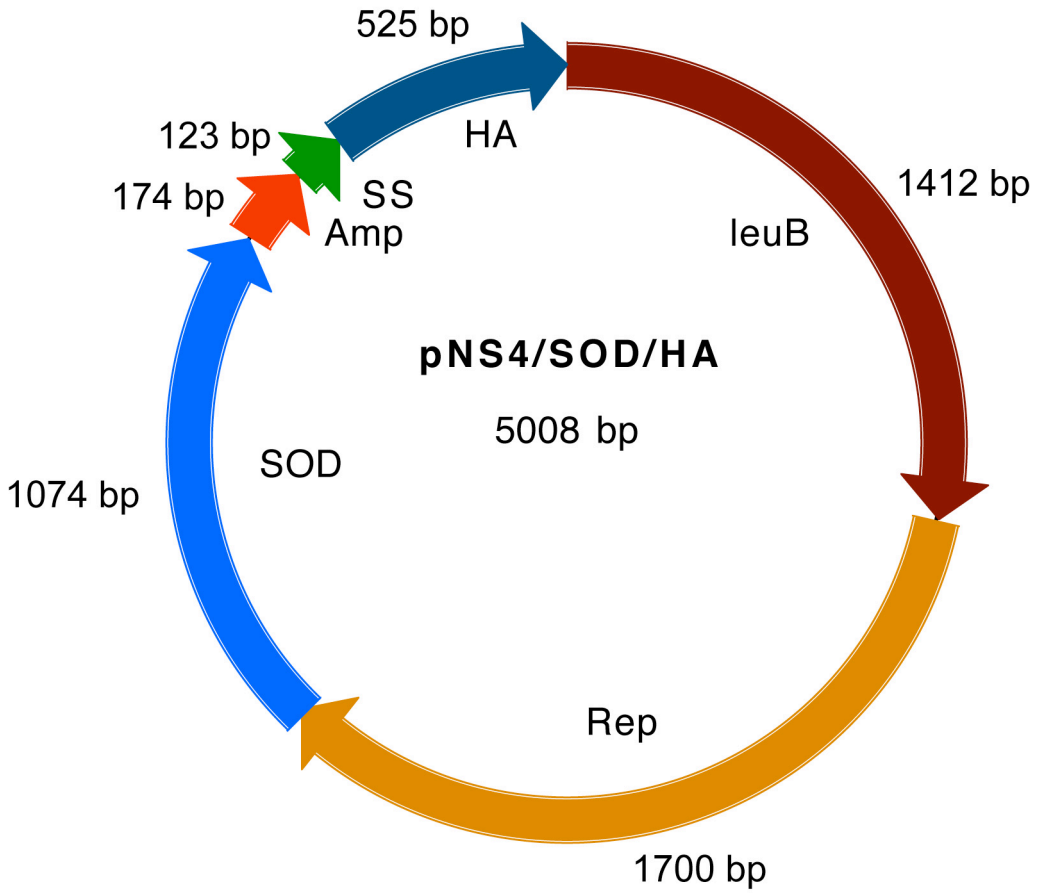

Fig. 1A Map of pNS4/SOD/HA

Rep – Origin of replication

leuB – intact *leuB* gene and its promoter region from *B. abortus*

SOD- Superoxide dismutase gene and its promoter region (*sodC)*

Amp – *bla* promoter

SS- *bla* signal sequence

HA- Globular domain region of hemagglutinin gene

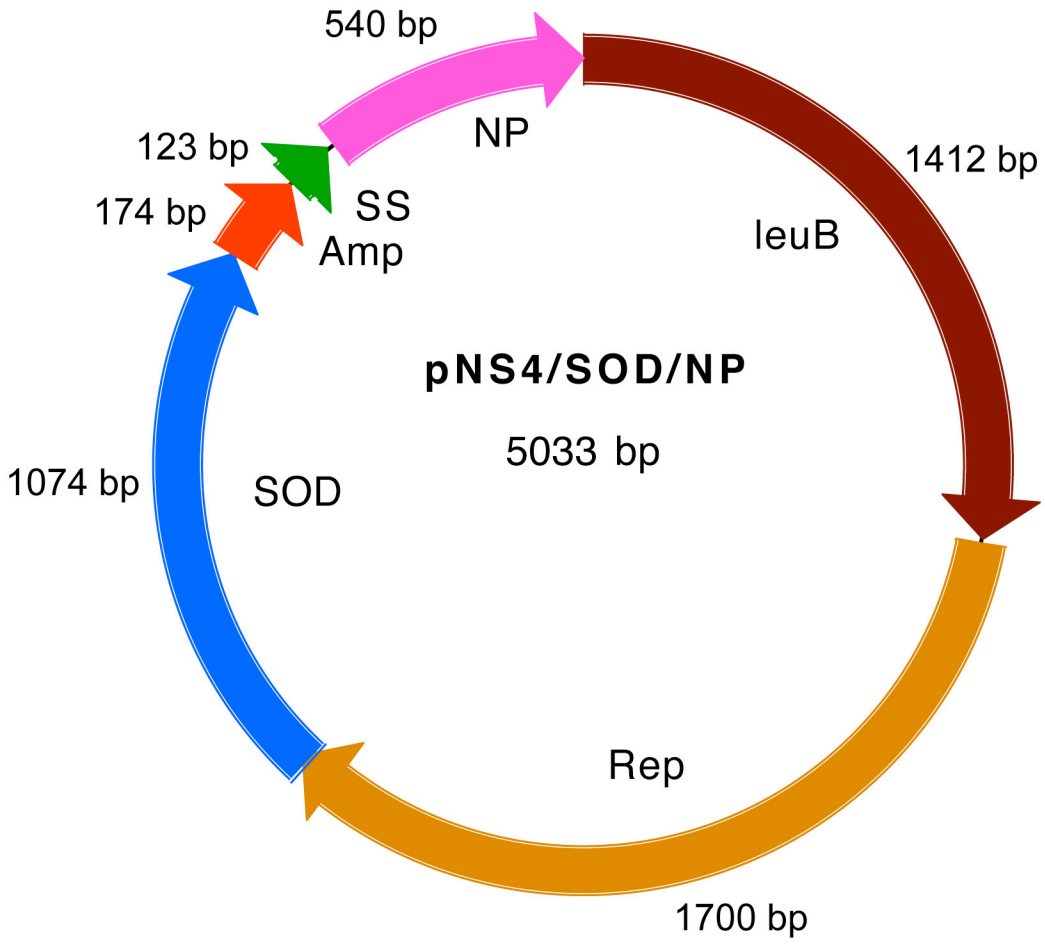

Fig. 1B Map of pNS4/SOD/NP

Rep – Origin of replication

leuB – intact *leuB* gene and its promoter region of *B. abortus*

SOD- Superoxide dismutase gene and its promoter region (*sodC)*

Amp – *bla* promoter

SS- *bla* signal sequence

NP- RNA groove region of nucleoprotein gene

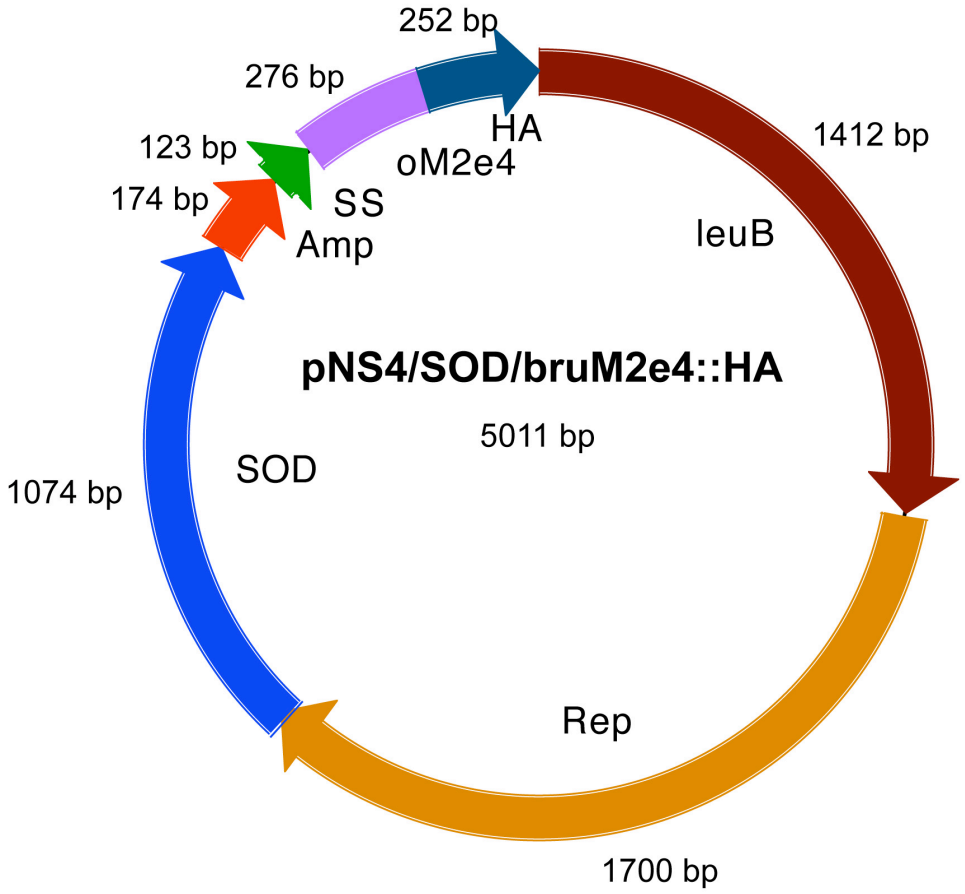

Fig. 1C Map of pNS4/SOD/bruM2e4::HA

Rep – Origin of replication

leuB – intact *leuB* gene and its promoter region of *B. abortus*

SOD- Superoxide dismutase gene and its promoter region (*sodC)*

Amp – *bla* promoter

SS- *bla* signal sequence

oM2e4- four repeats of ectodomain region of matrix protein (M2) optimized for *Brucella* codon usage

HA- Trans-membrane and cytoplasmic tail domain region of hemagglutinin gene

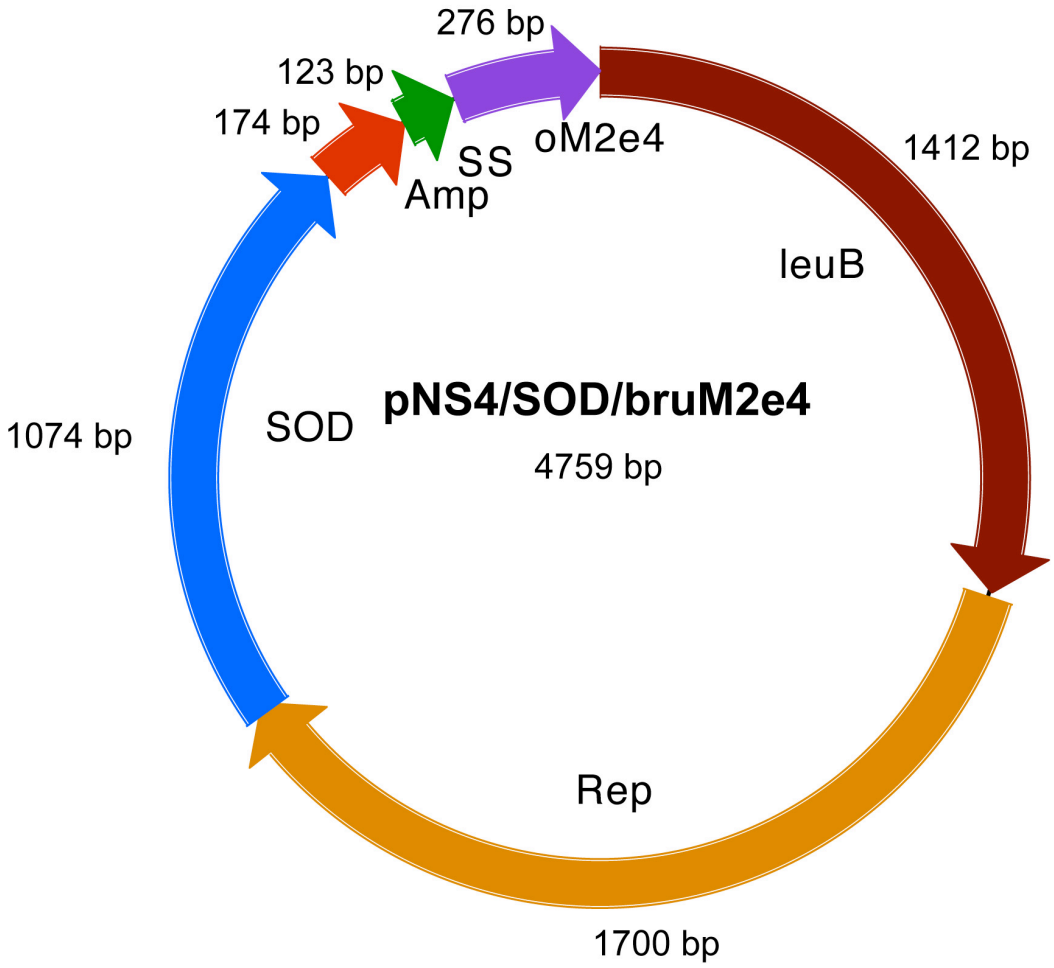

Fig. 1D Map of pNS4/SOD/bruM2e4

Rep – Origin of replication

leuB – intact *leuB* gene and its promoter region of *B. abortus*

SOD- Superoxide dismutase gene and its promoter region (*sodC)*

Amp – *bla* promoter

SS- *bla* signal sequence

oM2e4- four repeats of ectodomain region of matrix protein (M2) optimized for *Brucella* codon usage

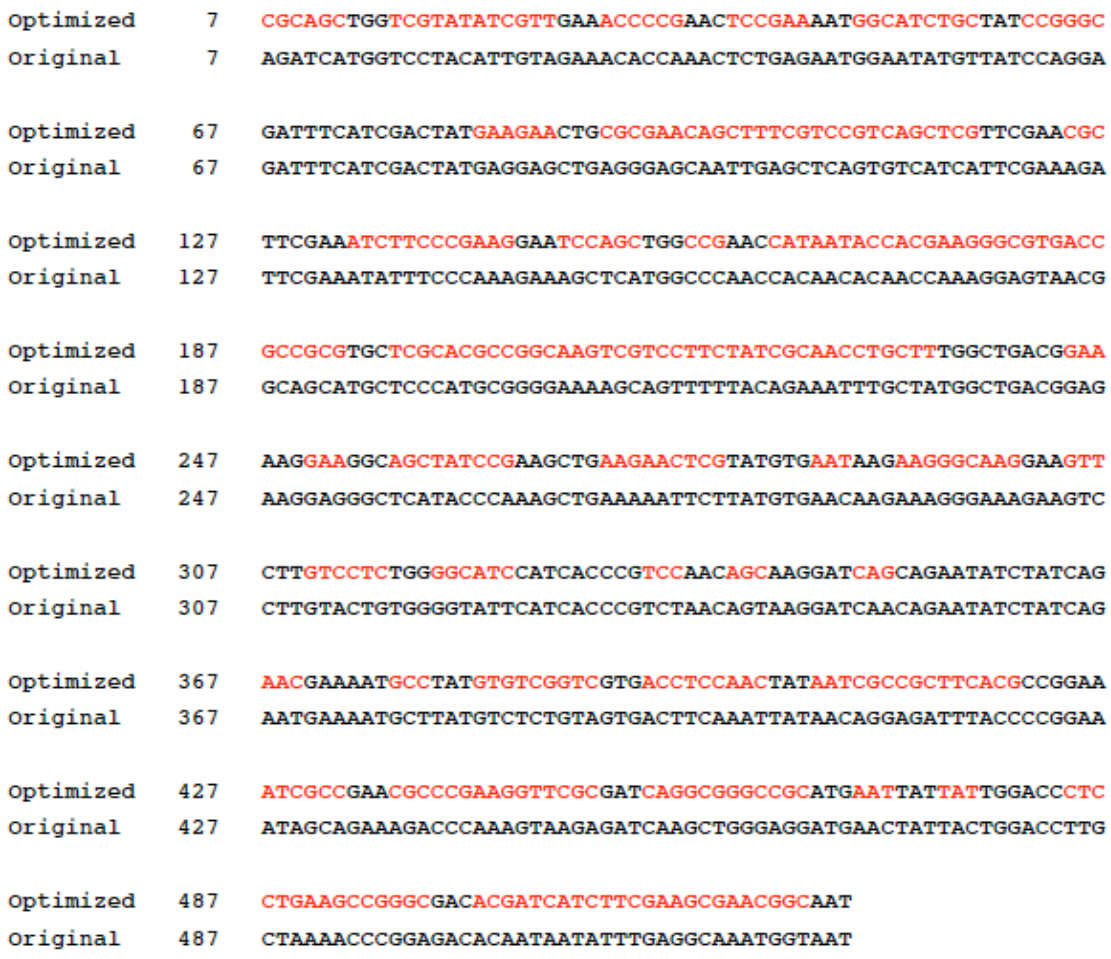

Fig. 2A. Sequence comparison of codon optimized and native HA gene

```
Optimized      7   ATGGCCACCAAGGGCACGAAGCGCTCCTATGAACAGATGGAAACCGATGGCGAACGCCAG
Original       7   ATGGCGACCAAAGGCACCAAACGATCTTACGAACAGATGGAGACTGATGGAGAACGCCAG

Optimized     67   AACGCCACGGAAATCCGCGCGAGCGTCGGCAAGATGATCGATGGCATCGGCCGCTTCTAT
Original      67   AATGCCACTGAAATCAGAGCATCTGTCGGAAAAATGATTGATGGAATTGGACGATTCTAC

Optimized    127   ATCCAGATGTGCACCGAACTGAAGCTTTCGGACTATGAAGGCCGCCTTATCCAGAACAGC
Original     127   ATCCAAATGTGCACCGAACTTAAACTCAGTGATTATGAGGGACGGCTGATTCAGAACAGC

Optimized    187   CTCACGATCGAACGCATGGTTCTTTCGGCCTTCGATGAACGCCGCAATAAGTATCTGGAA
Original     187   TTAACAATAGAGAGAATGGTGCTCTCTGCTTTTGACGAGAGGAGGAATAAATATCTAGAA

Optimized    247   GAACATCCGTCCGCGGGCAAGGACCCGAAGAAGACCGGCGGCCCGATCTATCGCCGCGTC
Original     247   GAACATCCCAGTGCGGGGAAAGATCCTAAGAAAACTGGAGGACCTATATACAGGAGAGTA

Optimized    307   GATGGCAAGTGGCGCCGCGAACTCATCCTGTATGACAAGGAAGAAATCCGCCGCATCTGG
Original     307   GATGGAAAGTGGAGGAGAGAACTCATCCTTTATGACAAAGAAGAAATAAGACGAATCTGG

Optimized    367   CGCCAGGCGAACAATGGCGATGACGCCACCGCGGGCCTCACGCATATGATGATCTGGCAC
Original     367   CGCCAAGCTAATAATGGTGACGATGCAACGGCTGGTCTGACTCACATGATGATCTGGCAC

Optimized    427   AGCAACCTGAATGATGCCACCTATCAGCGCACGCGCGCGCTGGTCCGCACCGGCATGGAC
Original     427   TCCAATTTGAATGATGCAACTTACCAGAGGACAAGAGCTCTTGTTCGCACAGGAATGGAT

Optimized    487   CCGCGCATGTGCTCGCTGATGCAGGGCTCCACGCTTCCGCGCCGCTCGGGCGCCGCGGGC

Original     487   CCCAGGATGTGCTCACTGATGCAGGGTTCAACCCTCCCTAGGAGGTCTGGGGCCGCAGGT

Optimized    547   GTG
Original     547   GTA
```

Fig. 2B. Sequence comparison of codon optimized and native NP gene

```
Optimized     7   TCGCTGCTTACCGAAGTGGAAACGCCGATCCGCAACGAATGGGGCTGCCGCTGCAATGAT
Original      7   TCCCTGCTGACCGAGGTGGAGACACCCATCCGCAACGAGTGGGGCTGCAGGTGTAATGAT

Optimized    67   TCGTCCGACTCCCTCCTGACCGAAGTTGAAACCCTGACGCGCAACGGCTGGGGCTGCCGC
Original     67   AGCTCCGACAGTCTCCTCACTGAGGTGGAGACACTGACCAGAAACGGATGGGGCTGCCGG

Optimized   127   TGCAGCGATAGCTCGGACTCGCTTCTCACGGAAGTCGAAACCCCGACGAAGAACGAATGG
Original    127   TGTTCCGATAGCTCCGACAGCCTGCTGACAGAGGTGGAGACCCCAACAAAGAATGAGTGG

Optimized   187   GAATGCCGCTGCAATGATTCCAGCGACAGCCTTCTGACCGAAGTGGAAACCCCGACGCGC
Original    187   GAGTGCCGCTGTAACGATTCCAGTGATAGCCTCCTCACAGAAGTCGAAACACCTACCAGG

Optimized   247   TCGGAGTGGGAGTGCCGCTGCTCGGATTCGTCCGACGGCTCCAACGGCACGTATGATTAT
Original    247   AGCGAGTGGGAGTGCAGATGCAGCGATAGCTCCGACGGAAGCAATGGCACATACGATTAC

Optimized   307   CCGAAGTATTCCGAAGAAAGCAAGCTGAATCGCGAAAAGATCGACGGCGTTAAGCTGGAA
Original    307   CCCAAATACTCCGAGGAGAGCAAGCTGAACCGGGAGAAAATCGACGGAGTGAAGCTGGAG

Optimized   367   TCCATGGGCGTCTATCAGATCCTTGCCATCTATAGCACCGTCGCGAGCTCGCTTGTCCTC
Original    367   TCCATGGGCGTGTACCAGATCCTGGCCATCTACAGCACCGTGGCCTCCAGCCTGGTGCTG

Optimized   427   CTGGTGTCCCTCGGCGCCATCAGCTTCTGGATGTGCTCGAACGGCTCCCTGCAGTGCCGC
Original    427   CTGGTGTCCCTGGGAGCCATCAGCTTTTGGATGTGCTCCAATGGCAGCCTGCAGTGTCGG

Optimized   487   ATCTGCATCTGAGAATTCAAGCTGGGCAAT
Original    487   ATCTGCATCTGAGAATTCAAGCTTGGTAAT
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Fig. 2C. Sequence comparison of codon optimized and native M2e4::HA gene

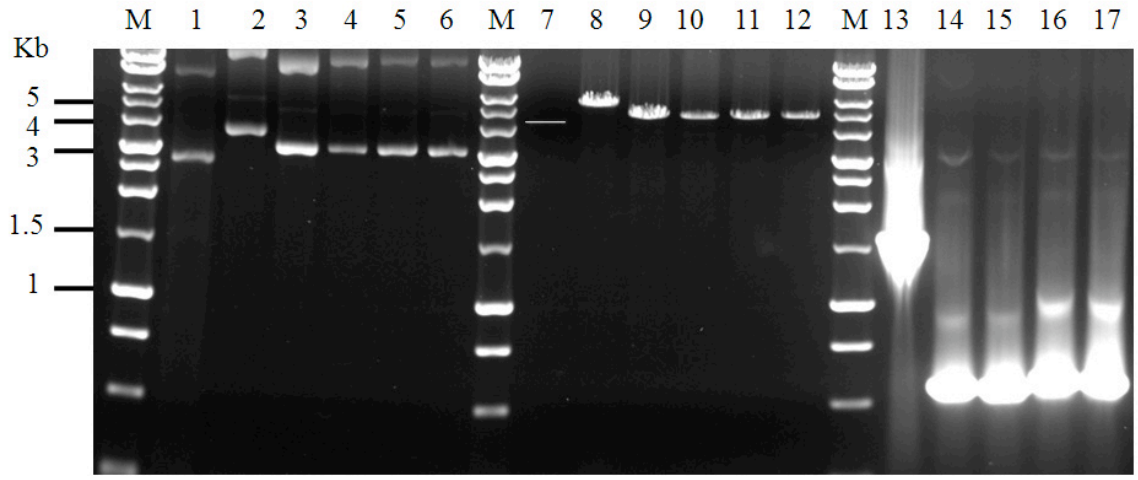

Fig. 3. Agarose gel showing the DNA isolated from strain RB51*leuB* transformed with corresponding plasmids: Lane- Sample; M- Standards, 1- pNS4/SOD/AmpSS (Circular), 2- pNS4/SOD/WboA (Circular), 3&4- pNS4/SOD/AmpSS::HA (circular - two different clones), 5&6- pNS4/SOD/AmpSS::NP (circular - two different clones), 7- pNS4/SOD/AmpSS (Linear) 4.07 kb, 8- pNS4/SOD/WboA (Linear) 5.36 kb, 9&10- pNS4/SOD/AmpSS::HA (Linear clones 3&4) 4.58 kb, 11&12- pNS4/SOD/AmpSS::NP (Linear clones 5&6) 4.61 kb, 13- PCR amplified *wboA* (1.55 kb), 14&15- PCR amplified HA from 3&4 (516 bp), 16&17- PCR amplified NP from 5&6 (540 bp).

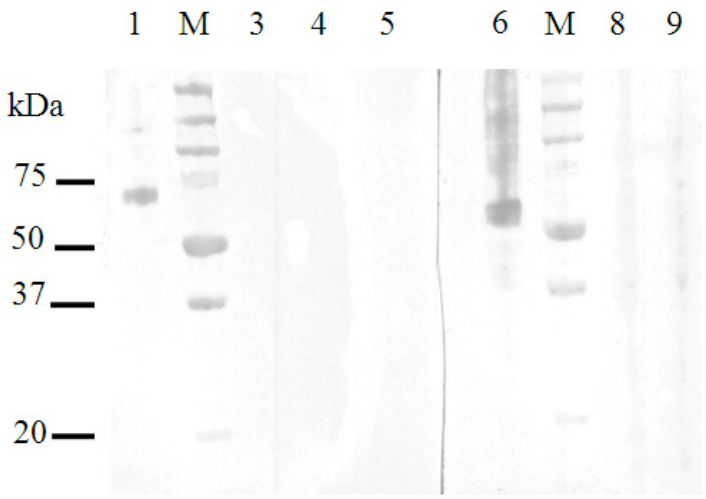

Fig. 4. **Immunoblot for screening optimized HA and NP expression:** Lane - Sample; M- Standards, 1- PR8 lysate (Positive control), 3- RB51*leuB*/pNS4/SOD, 4- RB51*leuB*/SOD/bruHA, 5- RB51*leuB*/SOD/bruM2e4::HA, 6- PR8 lysate (Positive control), 8- RB51*leuB*/SOD, 9- RB51*leuB*/SOD/bruNP. Lanes 1 to 5 screened using mouse HA antibodies and lanes 6 to 9 screened with goat RNP antibodies.

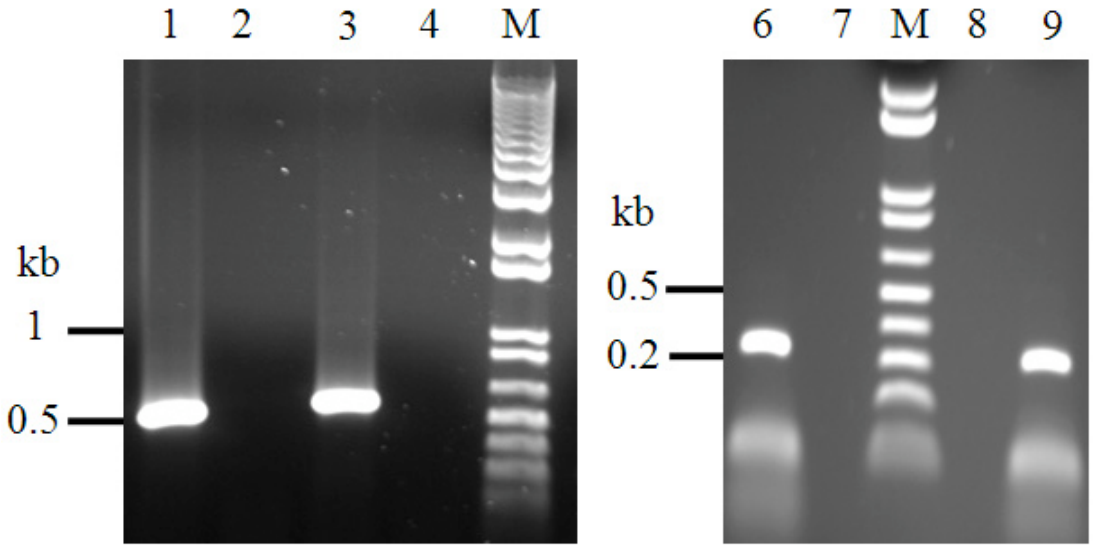

Fig. 5. PCR amplified fragments on agarose gel stained with ethidium bromide: Lane – source of RNA from which cDNA template was prepared; 1- RB51*leuB*/SOD/bruHA, 2- Negative control (for HA), 3- RB51*leuB* /SOD/bruNP, 4- Negative control (for NP), 6- RB51*leuB*/SOD/bruM2e4, 7- Negative Control (for M2e4), 8-Negative Control (M2e4), 9- RB51*leuB*/SOD/bruM2e4::HA, M- Molecular weight marker. (Size of fragments: HA-516 bp, NP- 540 bp, M2e4- 276 bp). Total RNA isolated from the constructs was used as template to produce cDNA. Then the cDNA were used as template to amplify the genes HA, NP and M2e4 using specific primers. For negative controls, the total RNA was used directly as template (as against cDNA) to rule out DNA contamination.

```
 30          40          50
ATGGTTTCTTAGACGTCAGGTGG
 M   V   S   *   T   S   G   G>

          60          70          80          90          100
CACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAA
  T   F   R   G   N   V   R   G   T   P   I   C   L   F   F   *>

        110         120         130         140         150
TACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTT
I   H   S   N   M   Y   P   L   M   R   Q   *   P   *   *   M   L>

        160         170         180         190         200
CAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGC
 Q   *   Y   *   K   R   K   S   M   S   I   Q   H   F   R   V   A>

        210         220         230         240         250
CCTTATTCCCTTTTTTGCGGCATCTTGCCTTCCTGTTTTTGCTCACCCAG
  L   I   P   F   F   A   A   S   C   L   P   V   F   A   H   P>

        260         270         280         290         300
AAACGCTGGTGAAAGTAAAAGATGCTGAACATCATCATCATCATCATGGA
E   T   L   V   K   V   K   D   A   E   H   H   H   H   H   H   G>

        310         320         330         340         350
TCCCGCAGCTGGTCGTATATCGTTGAAACCCCGAACTCCGAAAATGGCAT
 S   R   S   W   S   Y   I   V   E   T   P   N   S   E   N   G   I>

        360         370         380         390         400
CTGCTATCCGGGCGATTTCATCGACTATGAAGAACTGCGCGAACAGCTTT
  C   Y   P   G   D   F   I   D   Y   E   E   L   R   E   Q   L>

        410         420         430         440         450
CGTCCGTCAGCTCGTTCGAACGCTTCGAAATCTTCCCGAAGGAATCCAGC
S   S   V   S   S   F   E   R   F   E   I   F   P   K   E   S   S>

        460         470         480         490         500
TGGCCGAACCATAATACCACGAAGGGCGTGACCGCCGCGTGCTCGCACGC
 W   P   N   H   N   T   T   K   G   V   T   A   A   C   S   H   A>

        510         520         530         540         550
CGGCAAGTCGTCCTTCTATCGCAACCTGCTTTGGCTGACGGAAAAGGAAG
  G   K   S   S   F   Y   R   N   L   L   W   L   T   E   K   E>

        560         570         580         590         600
GCAGCTATCCGAAGCTGAAGAACTCGTATGTGAATAAGAAGGGCAAGGAA
G   S   Y   P   K   L   K   N   S   Y   V   N   K   K   G   K   E>

        610         620         630         640         650
GTTCTTGTCCTCTGGGGCATCCATCACCCGTCCAACAGCAAGGATCAGCA
 V   L   V   L   W   G   I   H   H   P   S   N   S   K   D   Q   Q>

        660         670         680         690         700
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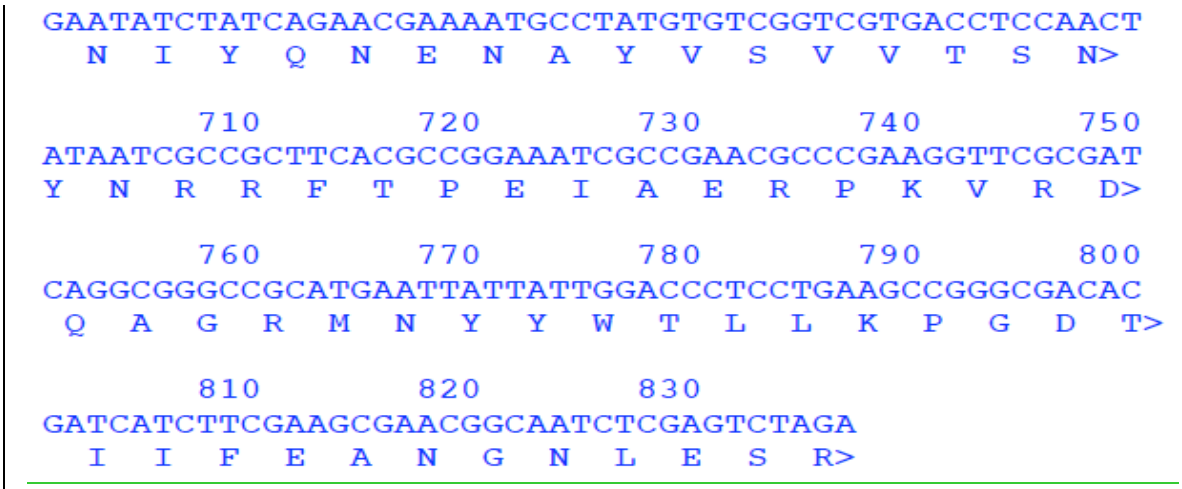

Fig. 6A AmpSS::bruHA

Color denomination for sequences:

Black: *bla* promoter region

Red: *bla* signal sequence

Green: 6x Histidine

Blue: bruHA

```
 30         40         50
ATGGTTTCTTAGACGTCAGGTGG
 M   V   S   *   T   S   G   G>

         60         70         80         90         100
CACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAA
  T   F   R   G   N   V   R   G   T   P   I   C   L   F   F   *>

        110        120        130        140        150
TACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTT
I   H   S   N   M   Y   P   L   M   R   Q   *   P   *   *   M   L>

        160        170        180        190        200
CAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGC
 Q   *   Y   *   K   R   K   S   M   S   I   Q   H   F   R   V   A>

        210        220        230        240        250
CCTTATTCCCTTTTTTGCGGCATCTTGCCTTCCTGTTTTTGCTCACCCAG
  L   I   P   F   F   A   A   S   C   L   P   V   F   A   H   P>

        260        270        280        290        300
AAACGCTGGTGAAAGTAAAAGATGCTGAACATCATCATCATCATCATAGA
E   T   L   V   K   V   K   D   A   E   H   H   H   H   H   H   R>

        310        320        330        340        350
TCTATGGCCACCAAGGGCACGAAGCGCTCCTATGAACAGATGGAAACCGA
 S   M   A   T   K   G   T   K   R   S   Y   E   Q   M   E   T   D>

        360        370        380        390        400
TGGCGAACGCCAGAACGCCACGGAAATCCGCGCGAGCGTCGGCAAGATGA
  G   E   R   Q   N   A   T   E   I   R   A   S   V   G   K   M>

        410        420        430        440        450
TCGATGGCATCGGCCGCTTCTATATCCAGATGTGCACCGAACTGAAGCTT
I   D   G   I   G   R   F   Y   I   Q   M   C   T   E   L   K   L>

        460        470        480        490        500
TCGGACTATGAAGGCCGCCTTATCCAGAACAGCCTCACGATCGAACGCAT
 S   D   Y   E   G   R   L   I   Q   N   S   L   T   I   E   R   M>

        510        520        530        540        550
GGTTCTTTCGGCCTTCGATGAACGCCGCAATAAGTATCTGGAAGAACATC
  V   L   S   A   F   D   E   R   R   N   K   Y   L   E   E   H>

        560        570        580        590        600
CGTCCGCGGGCAAGGACCCGAAGAAGACCGGCGGCCCGATCTATCGCCGC
P   S   A   G   K   D   P   K   K   T   G   G   P   I   Y   R   R>

        610        620        630        640        650
GTCGATGGCAAGTGGCGCCGCGAACTCATCCTGTATGACAAGGAAGAAAT
 V   D   G   K   W   R   R   E   L   I   L   Y   D   K   E   E   I>

        660        670        680        690        700
```

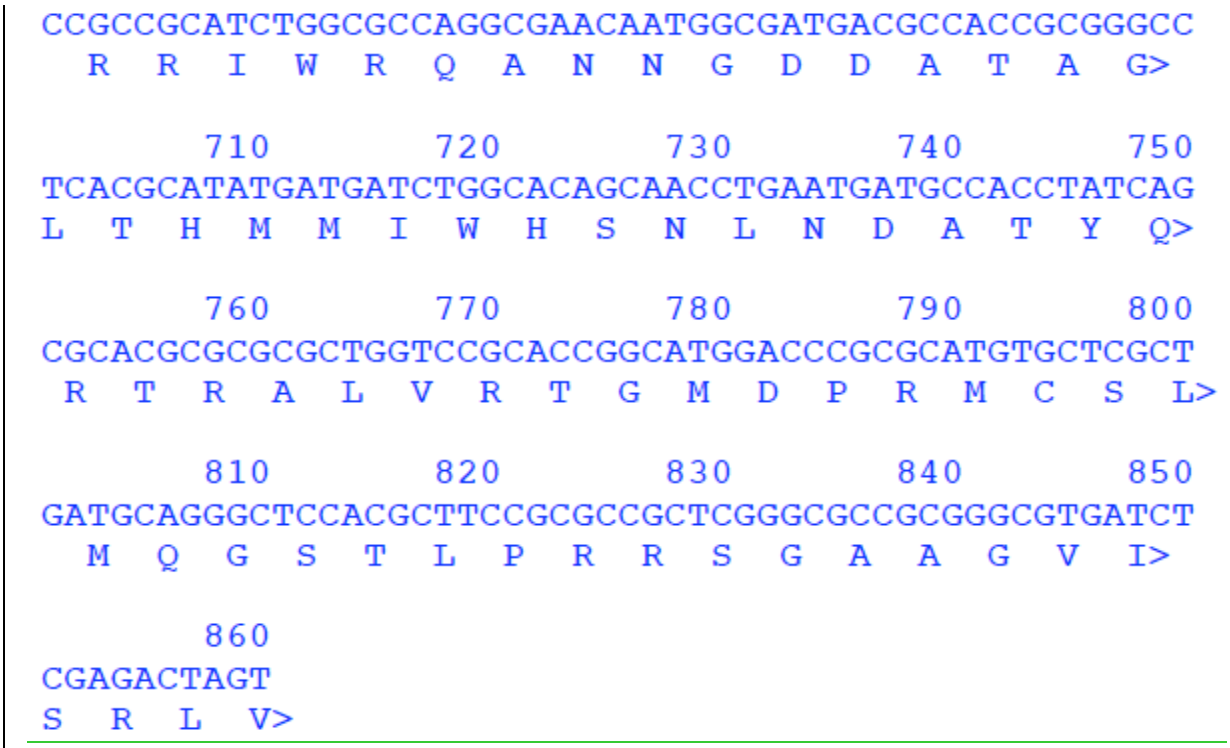

Fig. 6B AmpSS::bruNP

Color denomination for sequences:

Black: *bla* promoter region

Red: *bla* signal sequence

Green: 6x Histidine

Blue: bruNP

```
 30          40          50
ATGGTTTCTTAGACGTCAGGTGG
 M   V   S   *   T   S   G   G>

          60          70          80          90          100
CACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAA
  T   F   R   G   N   V   R   G   T   P   I   C   L   F   F   *>

        110         120         130         140         150
TACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTT
I   H   S   N   M   Y   P   L   M   R   Q   *   P   *   *   M   L>

        160         170         180         190         200
CAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGC
 Q   *   Y   *   K   R   K   S   M   S   I   Q   H   F   R   V   A>

        210         220         230         240         250
CCTTATTCCCTTTTTTGCGGCATCTTGCCTTCCTGTTTTTGCTCACCCAG
  L   I   P   F   F   A   A   S   C   L   P   V   F   A   H   P>

        260         270         280         290         300
AAACGCTGGTGAAAGTAAAAGATGCTGAACATCATCATCATCATCATGGA
E   T   L   V   K   V   K   D   A   E   H   H   H   H   H   H   G>

        310         320         330         340         350
TCCTCGCTGCTTACCGAAGTGGAAACGCCGATCCGCAACGAATGGGGCTG
 S   S   L   L   T   E   V   E   T   P   I   R   N   E   W   G   C>

        360         370         380         390         400
CCGCTGCAATGATTCGTCCGACTCCCTCCTGACCGAAGTTGAAACCCTGA
  R   C   N   D   S   S   D   S   L   L   T   E   V   E   T   L>

        410         420         430         440         450
CGCGCAACGGCTGGGGCTGCCGCTGCAGCGATAGCTCGGACTCGCTTCTC
T   R   N   G   W   G   C   R   C   S   D   S   S   D   S   L   L>

        460         470         480         490         500
ACGGAAGTCGAAACCCCGACGAAGAACGAATGGGAATGCCGCTGCAATGA
 T   E   V   E   T   P   T   K   N   E   W   E   C   R   C   N   D>

        510         520         530         540         550
TTCCAGCGACAGCCTTCTGACCGAAGTGGAAACCCCGACGCGCTCGGAGT
  S   S   D   S   L   L   T   E   V   E   T   P   T   R   S   E>

        560         570         580         590         600
GGGAGTGCCGCTGCTCGGATTCGTCCGACGGCTCCAACGGCACGTATGAT
W   E   C   R   C   S   D   S   S   D   G   S   N   G   T   Y   D>

        610         620         630         640         650
TATCCGAAGTATTCCGAAGAAAGCAAGCTGAATCGCGAAAAGATCGACGG
 Y   P   K   Y   S   E   E   S   K   L   N   R   E   K   I   D   G>

        660         670         680         690         700
```

Fig. 6C AmpSS::bruM2e4::HA

Color denomination for sequences:

Black: *bla* promoter region

Red: *bla* signal sequence

Green: 6x Histidine

Blue: bruM2e4::HA

```
 30         40         50
ATGGTTTCTTAGACGTCAGGTGG
 M   V   S   *   T   S   G   G>

         60         70         80         90         100
CACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAA
  T   F   R   G   N   V   R   G   T   P   I   C   L   F   F   *>

        110        120        130        140        150
TACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTT
I   H   S   N   M   Y   P   L   M   R   Q   *   P   *   *   M   L>

        160        170        180        190        200
CAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGC
 Q   *   Y   *   K   R   K   S   M   S   I   Q   H   F   R   V   A>

        210        220        230        240        250
CCTTATTCCCTTTTTTGCGGCATCTTGCCTTCCTGTTTTTGCTCACCCAG
  L   I   P   F   F   A   A   S   C   L   P   V   F   A   H   P>

        260        270        280        290        300
AAACGCTGGTGAAAGTAAAAGATGCTGAACATCATCATCATCATCATGGA
E   T   L   V   K   V   K   D   A   E   H   H   H   H   H   H   G>

        310        320        330        340        350
TCCTCGCTGCTTACCGAAGTGGAAACGCCGATCCGCAACGAATGGGGCTG
 S   S   L   L   T   E   V   E   T   P   I   R   N   E   W   G   C>

        360        370        380        390        400
CCGCTGCAATGATTCGTCCGACTCCCTCCTGACCGAAGTTGAAACCCTGA
  R   C   N   D   S   S   D   S   L   L   T   E   V   E   T   L>

        410        420        430        440        450
CGCGCAACGGCTGGGGCTGCCGCTGCAGCGATAGCTCGGACTCGCTTCTC
T   R   N   G   W   G   C   R   C   S   D   S   S   D   S   L   L>

        460        470        480        490        500
ACGGAAGTCGAAACCCCGACGAAGAACGAATGGGAATGCCGCTGCAATGA
 T   E   V   E   T   P   T   K   N   E   W   E   C   R   C   N   D>

        510        520        530        540        550
TTCCAGCGACAGCCTTCTGACCGAAGTGGAAACCCCGACGCGCTCGGAGT
  S   S   D   S   L   L   T   E   V   E   T   P   T   R   S   E>

        560        570        580        590
GGGAGTGCCGCTGCTCGGATTCGTCCGACTAATCTCAGATCTAGA
W   E   C   R   C   S   D   S   S   D   *   S   Q   I   *>
```

Fig. 6D AmpSS::M2e4

Color denomination for sequences:

Black: *bla* promoter region

Red: *bla* signal sequence

Green: 6x Histidine

Blue: bruM2e4

Overall Summary and Conclusion

Vaccines have proven to be one of the most effective tools in eradication and containment of devastating diseases in both humans and animals. As per the concept "One Health", disease prevention in animals is an essential strategy towards achieving the goal of a healthier world. Development and use of multivalent vaccines in veterinary medicine has proven to be a more cost effective infection prevention technique against multiple diseases. The USDA approved vaccine *B. abortus* strain RB51 is used against bovine brucellosis. This vaccine has shown to elicit protective immune response in mice under laboratory conditions against those antigens (homologous and heterologous) that are over-expressed on a plasmid that is compatible with *Brucella spp*. To release a recombinant multivalent vaccine into field environment, traits such as antibiotic resistance cassette (used as plasmid markers) are discouraged. To address the regulations, in this study, an antibiotic resistance free plasmid over-expression system was developed for use in strain RB51. A leucine auxotroph of strain RB51 and the *leuB* complementing plasmid pNS4 were constructed as an alternative to antibiotic resistance marked over-expression system. In this balanced lethal system, strain RB51*leuB* cannot grow in leucine deficient conditions unless complemented by the intact *leuB* gene in pNS4. The leucine auxotrophic property was utilized for *in vitro* manipulation of the plasmid and selection of recombinant bacterial clones. The attenuation levels and protective ability (against *B. abortus* challenge) of the leucine auxotroph strain were similar to the parent strain RB51. The plasmid pNS4 was able to over-express a model antigen GFP on the strain RB51*leuB* platform both *in vitro* and in mice.

The strain RB51*leuB* and pNS4 were able to over-express *Brucella* derived protective antigens: SOD (superoxide dismutase), ribosomal protein L7/L12 and O-antigen. The recombinant candidate vaccine strains were able to protect mice significantly from a virulent *B. suis* challenge when compared to unvaccinated mice. The mice vaccinated with strain RB51*leuB* over-expressing either SOD alone or SOD and L7/L12 were protected at similar levels. Over-expression of L7/L12 along with SOD did not enhance protection. Among the different vaccine groups, the strain RB51*leuB*/SOD/WboA protected mice significantly compared with the control as well as other candidate vaccine strains. This strain could be a potential candidate for use against brucellosis in swine and should be actively pursued for next stage of vaccine trials.

Influenza virus antigens, hemagglutinin (HA) and nucleoprotein (NP) cloned onto pNS4 were found not to be expressing in the *B. abortus* RB51*leuB* platform. Even after the codons of the viral antigens (HA and NP) were optimized to *Brucella* codon usage, no expression of antigens was detected in strain RB51*leuB*. Along with HA and NP, the sequence of the ectodomain of the influenza matrix protein (M2e) was also optimized. Again, no expression was detected of the M2e tetramer that contains M2e sequences from four different strains. Further refinements are needed for over-expressing viral antigens on a *Brucella* platform.

In conclusion, strain RB51*leuB*/pNS4 is as effective as its parent strain in eliciting protective immune responses against the over-expressed homologous antigens and thereby can be used to develop environmentally safe multivalent vaccines that would comply with regulations.