

INTRODUCTION

The first disease for which a bacterial agent was found to be the cause was anthrax (Greek meaning coal for the characteristic eschars found on the skin) (11,19). The disease is thought to have been the cause of the fifth and sixth plagues that attacked the livestock and people of Egypt in Exodus 9 (5,11,17).

Casimir Davaine first isolated the bacterium, *Bacillus anthracis*, in 1850 (11). In 1876, Robert Koch developed a method for culturing pure *B. anthracis* and in 1881, Louis Pasteur created the first major vaccine against the disease in livestock (5,11). Despite the existence of vaccines against the disease since the 1870's, anthrax remains a serious threat to livestock and even humans in the developing countries of Asia, Africa and South America (19). *B. anthracis*, the causative bacterial agent of anthrax, is believed to be part of the biological arsenals of at least ten nations. With the current world environment and the unpredictable threat of terrorism, it would seem prudent to include biological warfare defense measures into our national defense arsenals (8,11).

The bacteria in the genus *Bacillus* are characterized as large, gram-positive, rod-shaped organisms (31). *B. anthracis* occurs singly or in pairs in tissue, and in culture appear in long strings giving a classic "boxcar" look. The anthrax bacterium is never motile (19). *B. anthracis* is a spore-forming bacterium whose spores can survive in dry form for indefinite periods of time and then be released into the atmosphere with the capability of causing disease (8). Spore formation occurs after host death. *B. anthracis* spores are highly resistant to environmental changes such as temperature extremes, ultraviolet exposure, moisture or the lack thereof, and chemical treatment (17,19). It is the highly resistant nature of the spores of *B. anthracis* that aids in the persistence of the bacterial disease in an area (17).

The disease can take three forms: cutaneous, respiratory or gastrointestinal, depending upon the route of spore entry (17,38). The latter two forms of the anthrax are the

most fatal and most rare. When spores enter the host phagocytes engulf them. Nonvirulent strains are destroyed while virulent strains remain free in the cell's cytoplasm and germinate (19). The resulting vegetative cells replicate quickly; these cells enter the bloodstream and result in septicemia (17,19). However, after inhalation, the spores may remain unchanged in the lymph nodes, spleen, and bone marrow for indefinite periods of time (55).

Bacillus anthracis has two known virulence factors, both of which are plasmid-encoded (31). The first virulence factor is the poly-D-glutamic acid capsule. The capsule has anti-phagocytic properties, which enable the bacterium to resist a host's defenses (48). The genes encoding the capsule are located on the 90 kilobase (kb) pXO2 plasmid (3).

The second known virulence factor is the three component exotoxin consisting of the protective antigen (PA), lethal factor (LF), and edema factor (EF) (64). All of the proteins, which collectively make up the anthrax toxin, are encoded by a 175 kb plasmid called pXO1 (66). The anthrax toxin works on the common A-B model of bacterial exotoxin activity. This model requires a B or binding moiety and an A or enzymatic moiety for toxic activity to occur. Many common intracellularly acting toxins such as cholera, diphtheria, pertussis and botulinum toxin are explainable using the A-B model (43,69). However, the anthrax toxin is unique in that it consists of one B component and two A components (Figure 1) (4,43,65,69). LF is thought to destroy host cells by disrupting the mitogen-activated protein kinase pathway (18,44). EF is a calcium and calmodulin dependent adenylyl cyclase, which causes cellular edema in the host by

A-B Model of Anthrax Toxin

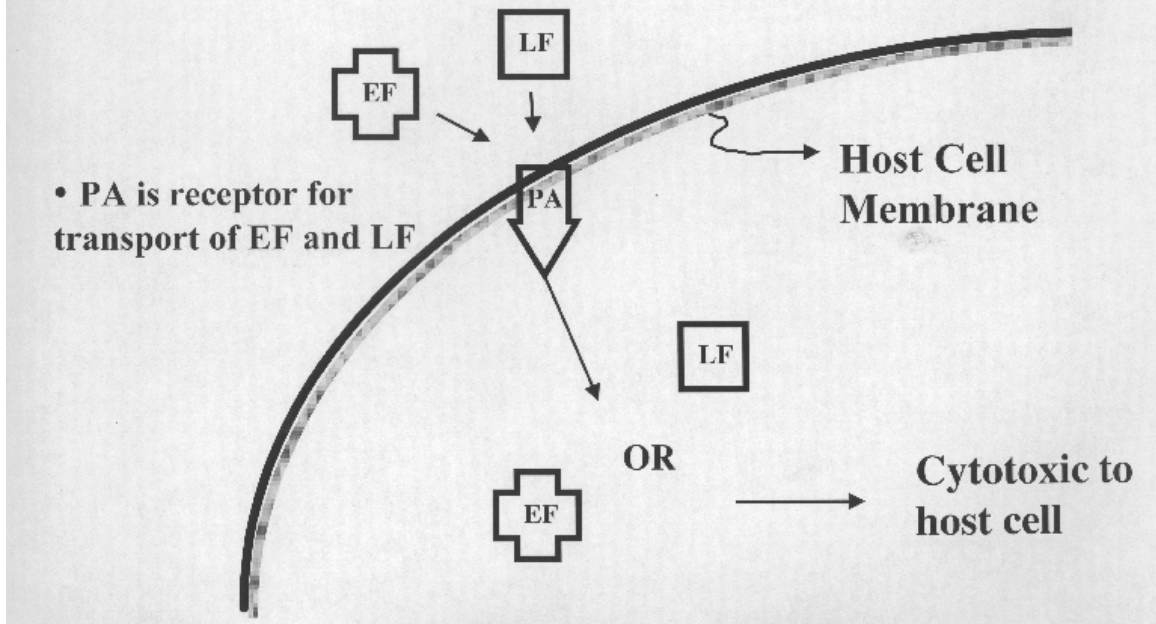


Figure 1: The A-B model of the anthrax toxin. The A-B model of anthrax consists of a B moiety and 2 A moieties. The PA protein binds to a cell receptor then acts as the effector for the binding and internalization of EF and LF. Since there are two A moieties, EF and LF bind competitively to PA.

increasing cAMP levels (44,65). PA is a protein that binds to host cell surface receptors (64). Once seven PA molecules have bound to a receptor, a channel is formed in the cell wall that facilitates entry of LF or EF into the cell's cytoplasm (44,64). Alone PA, LF, and EF are biologically inactive as toxins (38,63).

For toxic activity to occur, PA must be present. Once PA has become bound to the host cell receptor, it is cleaved from an 83kDa protein into a 63kDa protein by a cell surface protease (4). This cleavage activates PA, presumably by triggering a conformational change in the protein (65). Activation of PA allows EF or LF to competitively bind and be internalized by the host cell (4). Without PA, EF and LF could not gain access to the cell to exert their toxic effect (48).

PA is a very important component of the anthrax toxin for another reason: this protein plays a major role in anthrax immunity after both immunization and infection (48). A number of antigens of *B. anthracis* have been studied for their ability to induce protective immunity to the disease. Of the known antigens including the capsule, S-layer, surface polysaccharides and other proteins, only those proteins, which together make up the anthrax toxin, cause detectable production of antibodies (38,51,60). Of the three proteins, EF, LF and PA, only PA elicits antibodies which are protective against the disease (36,52,64). This immunity is believed to be brought about by neutralizing the activity of the anthrax toxin (20). Antibodies to PA will either block the protein from binding to host cell receptors or once bound will block the action of protease cleavage. Either situation renders PA biologically inactive. Without active PA bound to the cell, EF and LF cannot enter the cell. Thus, the anthrax toxin's influence on the host is halted (51).

Therefore, since PA is the only antigen known to induce protective antibodies against anthrax, the protein has become the main focus of anthrax vaccine research (60,24). PA, when produced in the absence of LF and EF, has been shown to be capable of producing effective protection both as a purified protein and when used in a recombinant or attenuated vaccine (60). However, protection studies have shown that high antibody titers to

PA do not correlate to strong protection (20,61). In fact, the veterinary live spore vaccine produced from the Sterne strain of *B. anthracis*, gives better and more prolonged protection against infection by the bacterium than merely adjuvanted PA (20,48,56,57,60,61). The knowledge that spore vaccines confer stronger, more reliable immunity to the disease seems to point to a role for cell-mediated immunity (CMI) in protection of the host (14,38,41,48,60,62).

The anthrax vaccine licensed for human use in the United States was developed by the Michigan Department of Public Health (MDPH) and is prepared by the Michigan Biological Products Institute (36). The AVA (anthrax vaccine adsorbed) is a subunit vaccine in that it is a cell-free extract. The vaccine is an aluminum hydroxide-adsorbed sterile culture filtrate containing mostly PA (25). The filtrate is derived from a fermentor culture of a non-encapsulated, toxigenic strain of *B. anthracis* called V77-NPI-R (25,26). The vaccine strain is cultured in a synthetic medium that promotes synthesis of PA preferentially over other proteins during the growth phase (36).

The human anthrax vaccine has several negative characteristics. For full immunity, a course of six immunizations over eighteen months followed by annual boosters is required (26,36). Local reactions have been noted in those receiving this vaccine in figures as high as 35%; this local reaction can take the form of local pain, redness and inflammation (25,26,51). Another drawback of this vaccine is the apparent inability of the vaccine to fully protect guinea pigs from aerosol challenge with highly virulent strains of *B. anthracis*, even after a full course of immunizations (14,24,25,26). This last problem could be due to the assumption that only a humoral response mainly to the PA present is enough to confer protection as opposed to a CMI or humoral response to other anthrax proteins is required for full protection (14,26).

The licensed vaccine for veterinary use is a live spore preparation produced by the Colorado Serum Company (10). The strain of anthrax used in this vaccine was developed by Sterne in the 1930's (57,58,60). The *B. anthracis* Sterne strain is non-encapsulated and

attenuated (24). The Sterne strain lacks the pXO2 plasmid encoding the capsule but retains the pXO1 plasmid encoding the exotoxin. Various studies have shown this vaccine to be superior to cell-free vaccines in affording protection even against highly virulent strains of anthrax. This protection is possibly due to the induction of CMI response in the animal (26,38,41,60). The live spore vaccine requires only one initial immunization (two in areas where the disease is endemic) followed by yearly boosters for full immunity (10). However, this anthrax vaccine has two negative characteristics. The strain used in the veterinary vaccine retains the ability to cause local necrosis at the site of injection and to cause disease in certain animal species (24,38).

It is this possible incidence of disease, albeit a rare occurrence which keeps Western nations from using a live vaccine to immunize humans against anthrax (38). However, the former USSR developed a live spore vaccine for human use. This vaccine is derived from a Sterne-like strain known as STI.

The STI vaccine was licensed for safe administration by scarification and subcutaneous inoculation initially (48,49). Later, after clinical trials, the vaccine was also judged to be safe and effective if given by aerosol route (38,48). Adverse effects of this vaccine seem to be limited to a transient elevation in temperature and, in the case of subcutaneous injection, a slight swelling at site of inoculation (49).

Efficacy of the STI vaccine is judged by the anthraxin test. Anthraxin is a heat-stable polysaccharide-protein-DNA complex derived from a non-encapsulated strain of *B. anthracis* (48). This complex does not contain capsular or toxigenic material produced by *B. anthracis* (48). The anthraxin skin test works on the principle of the tuberculin skin test and is based on cell mediated immunity (47,49,50). The anthraxin complex is injected intradermally and read 24 hours later. Positive reactors exhibit local erythema, with a diameter of at least 8mm, and induration, that lasts for 48 hours (50). This test reliably identifies vaccine-induced immunity in guinea pigs, sheep, and humans, as well as human

patients with histories of anthrax 20-30 years in the past, well after antibodies against *B. anthracis* proteins have disappeared (48).

While knowledge of the role of CMI in anthrax immunity is scarce, recent studies have demonstrated that live vaccines (not necessarily live spores) afford better protection than the chemical ones (48). However, patients and health care workers are reluctant to use a live spore anthrax vaccine, even if the strain is avirulent, for fear of its conversion to the virulent form. Therefore, studies of chemical PA vaccines adjuvanted with substances that elicit nonspecific CMI responses are being performed.

PA alone, with no adjuvant, is unable to effectively protect against a spore challenge. This is especially true if the protein becomes degraded. Proteolytic digestion of PA into fragments smaller than the biologically active 63kDa size, yield protein products which are incapable of inducing antibodies able to provide protection (40). In order for PA to induce protective antibodies, the protein must be of the 63-83kDa size.

In studies comparing injection of PA alone or PA combined with some adjuvant, either chemical or bacterial in origin, PA alone was less efficacious than any combination by a factor of about 4 (27). The least efficacious adjuvant was saponin; the same used in the AVA vaccine. Those chemical and bacterial product adjuvants, which stimulate CMI, confer higher levels of protection than those which only elicit humoral response (28). In fact, PA combinations using bacterial products as adjuvants conferred superior protection over those combined with chemical adjuvants (27). Due to this observation, PA has been expressed in several different bacterial and viral species such as *Echerichia coli*, *Salmonella typhimurium*, *Bacillus subtilis*, and vaccinia virus. These constructs have been tested for vaccination efficacy against anthrax spore challenge (2).

It is hoped that these new live recombinant bacterial strains expressing PA could be used as potential live vaccines against anthrax. The hypothesis is that these live attenuated bacterial strains will be able to induce a CMI response that will enhance the protective

abilities of PA against spore challenge. Previous studies have suggested a need for both humoral and CMI activation to achieve superior immunity against *B. anthracis* (27,28,29).

The first recombinant bacterial strain to express PA was *E. coli*. Drs. Leppla and Vodkin cloned the *pag* gene into a plasmid vector, transformed *E. coli* and checked for recombinants using Western blot and ELISA (64). Several colonies producing PA were identified, however, the level of protein expression was extremely low and the PA synthesized was degraded almost immediately (47). Until recently, one was able to isolate PA from *E. coli*, but it was badly degraded and functionally inactive. In 1999, researchers in India using *E. coli* were able to purify recombinant PA of correct size and functionally active (22). This recombinant protein will undergo vaccine trials which will be the first such trials using *E. coli*.

PA has been expressed in the bacterial strain *S. typhimurium*. The recombinant PA produced by *Salmonella* seems to be more stable than that produced by *E. coli* and is functionally and immunologically active. *S. typhimurium* expressing PA was used in a vaccine trial comparing its efficacy as a live recombinant vaccine against PA protein combined with adjuvants. In this trial, the live vaccine had an efficacy rate of 33% when given orally. This is comparable to the efficacy rate of adjuvanted PA, which conferred 37% protection (14). Further studies into the usefulness of this recombinant strain are being done.

In addition to expressing PA in bacteria, the protein has also been expressed in both vaccinia virus and baculovirus. PA was expressed in the WR and Connaught strains of vaccinia virus (23). Vaccine trials of these two recombinant strains in mice showed that WR-PA conferred 60% protection, while the Connaught-PA failed to protect at all (23). The baculovirus-PA strain had a 50% efficacy rate. These results show that PA expressed in virus is intact, functional and protective. The new constructs could be useful in future vaccine development (23).

Perhaps one of the best characterized recombinant bacterial strains expressing PA is *B. subtilis*. *B. subtilis* clones have been shown to produce PA in levels equal to or greater than those seen in *B. anthracis* (37,52). Expression of PA in this strain seems to be very stable and functionally active (1,30). Vaccination trials utilizing live *B. subtilis* also appear to be very promising (1,26,30). Clones expressing PA have been compared to both the AVA and live spore vaccines in efficacy studies. Results have shown that the *B. subtilis* clones have efficacy equal to the live spore vaccine and better than the AVA vaccine (26,30).

Literature searches have yielded many references to papers stating that live vaccines, whether they be natural or recombinant in nature, tend to confer better immunity against anthrax than subunit/adjuvanted vaccines do. For this reason, we have decided to express the *B. anthracis* PA gene in *Brucella abortus* vaccine strain RB51. Successful expression of this protein in strain RB51 would possibly enable us to create a vaccine which protects against two economically disastrous bacterial diseases: anthrax and brucellosis.

Brucellosis is one of several zoonotic diseases that can occur in both human and animal; another example is anthrax. In animals, the most obvious sign of disease is the appearance of abortion (21). Human brucellosis is characterized mainly by undulant fever and malaise (54).

Brucella infected animals and humans often present with widespread granulomas in areas such as the lymph nodes, bone marrow, liver and spleen. Abscesses have also been observed in bone, liver spleen, kidney and the brain (12). Placentitis is often seen in pregnant animals, with resulting abortion. Due to the frequent involvement of the mammary glands, *Brucella* is usually shed in milk. The organism is also present in aborted fetuses, fetal membranes and uterine discharge (21).

Natural transmission of *Brucella* is thought to occur by ingestion. This is due to the large numbers of organisms present in aborted tissue (21). Transmission occurs when animals ingest contaminated food and water or lick a recently aborted fetus (12). Infection

may also result in humans by ingesting infected milk or other dairy product. Also, *Brucella* can enter through abraded skin or contact with mucous membranes (6).

Due to the extensive economic damage *Brucella* infection can bring, eradication of the disease worldwide is very important. Vaccination is an effective means of protecting animals that have not already been exposed to the disease (21). However, since treatment of infected animals with antibiotics is not economically feasible, the U.S. has adopted the test-and-slaughter policy. Animals that give a positive reaction in the serum agglutination and other tests are separated from the herd and slaughtered. The remaining cattle in the herd are vaccinated and tested periodically for any additional positive reactors. A herd is considered brucellosis free if it tests negative 2 or 3 successive times in the serum agglutination test (21).

The causative agent of brucellosis is a bacterial strain from the genus *Brucella*. The genus consists of six main species: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* (9). Classification is based upon differences in pathogenicity and natural host. The major agents of brucellosis are the *Brucella* species: *B. abortus*, *B. melitensis*, and *B. suis* (21).

Brucella are gram-negative, non-motile, facultative intracellular bacteria (9). These bacteria are able to survive and even multiply inside the macrophage (21). *Brucella* do not have a protective capsule and do not produce spores. The various species of the genus *Brucella* share a close taxonomic relationship which extends even to the genetic level. All genetic information for *Brucella* organisms is chromosomally encoded that share at least a 90% homology across the genus (6,54). Unlike other bacteria, *Brucella* do not appear to harbor plasmids (54).

Several virulence factors aid *Brucella* in their survival inside macrophages and other cells. The first and probably most important factor is the presence of the O-side chain on

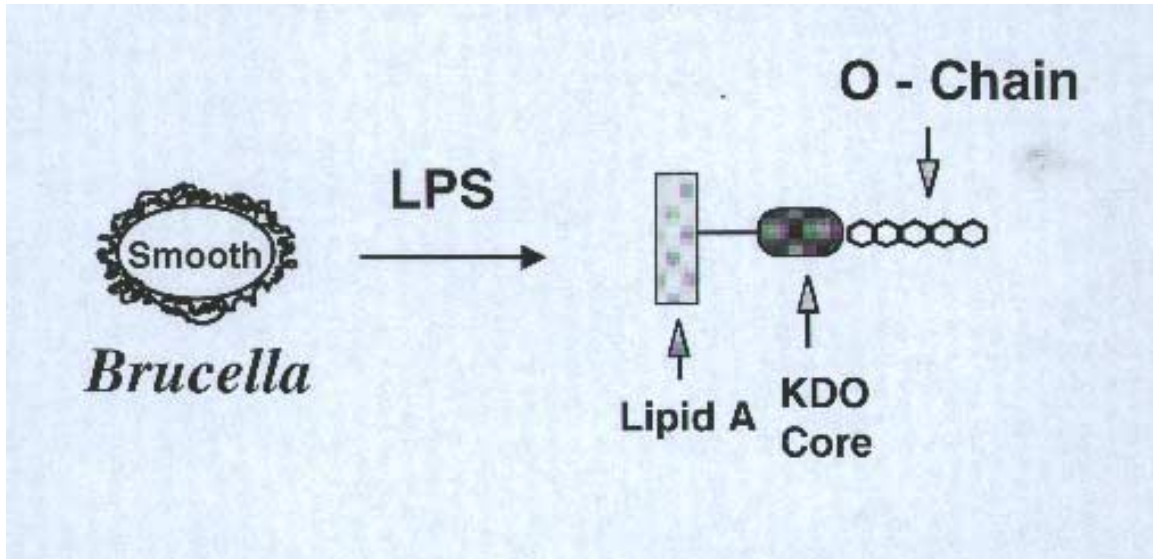


Figure 2: The LPS of smooth strains of species of the genus *Brucella*.

the lipopolysaccharide (LPS) of smooth strains. The O-side chain is the most exposed antigen structure seen in *Brucella* (9). The O-side chain of *Brucella* LPS induces a humoral response that is somewhat protective in mice but not in cattle (Figure 2) (12). In fact, it appears that production of antibodies of certain subisotypes against *Brucella* interferes with complement activation. Interference in this process could then allow *Brucella* to survive longer in cattle and set up a persistent infection (54).

Several strains of *Brucella* are naturally of the smooth morphology. These species include *B. abortus*, *B. melitensis* and *B. suis*, although these strains can also exhibit rough phenotype as well (9). *B. ovis* and *B. canis* occur naturally as rough species. Rough colony morphology in *Brucella* denotes the lack of the O-side chain on the LPS (12). Therefore, these strains do not cause induction of antibodies against the O-side chain, which interfere with serodiagnosis.

Elucidation of the factors, especially those that induce highly protective responses, is important in the development of effective vaccines. Also, the development of a highly efficacious vaccine means that it possesses several characteristics, including induction of long-term immunity, minimal interference with diagnostic tests, easy production and storage, poses no danger to the recipient, low cost and maintains a high level of quality (38). Vaccination of animals with live *Brucella* induces both humoral and CMI responses. The strength and duration of these responses depends highly upon the antigen used to induce the reaction and other factors such as dose and route of vaccination (38).

Several vaccines against *Brucella* have been developed for use in humans and animals and will be discussed here. As is the case with immunization against anthrax, the fight against brucellosis involves the use of both live and killed/subunit vaccines.

Immunization studies in laboratory animals using the subunit or killed vaccines against *Brucella* have not been promising. Examples of these dead vaccines are *B. abortus* strain 45/20 and *B. melitensis* H38.

B. abortus strain 45/20 is an adjuvanted vaccine of dead whole cells exhibiting the rough phenotype. No O-side chain is present in the preparation and therefore, the vaccine does not cause interference in serum agglutination tests. Strain 45/20 requires 2 initial doses, 6-12 weeks apart followed by annual boosters (21). Local reaction at site of injection may occur but killed strain 45/20 has not been shown to induce abortion (38).

B. melitensis H38 is an adjuvanted vaccine first developed for use in sheep and goats. This vaccine is composed of formol-killed whole cells of the smooth phenotype. Strain H38 induced immunity has not been well characterized. The vaccine is also shown to cause local reaction at site of inoculation and due to the presence of O-side chain in the preparation, and antibodies against the O-side chain interferes with serum testing (21,38).

Several live vaccines for use in animals have been developed worldwide with varying degrees of success in protecting against brucellosis. *B. abortus* strain 104-M isolated from a cow was developed in the former USSR. Virulence, immunogenicity and antigenic structure are reported to be stable (38). *B. suis* strain 2, developed in China, consists of an attenuated smooth strain of biovar 1 of *B. suis*. This vaccine has been used in several animal species; immunization with strain 2 does not seem to induce abortion in pregnant animals (38). Serologic interference by the vaccine seems to be low and short-lived.

The three most widely used live vaccines against brucellosis are *B. melitensis* Rev 1, *B. abortus* strain 19, and *B. abortus* RB51. *B. melitensis* Rev 1 was developed for use in sheep and goats and was derived from a virulent smooth strain of *B. melitensis* (12,21). The vaccine strain exhibits reduced virulence and induces effective immune responses in vaccinated animals. Vaccination is performed in young sheep and goats subcutaneously; a lower dose can be used to immunize adult animals. The vaccine induces serum antibodies which are persistent; strain Rev 1 may induce abortion in pregnant animals (38).

B. abortus strain 19 is a viable smooth strain used in cattle since the 1930's. The positive and negative characteristics of this vaccine are well known. Strain 19 is primarily used for calf-hood immunizations but vaccination of adults is also possible (38). Normally,

this vaccine is given in one dose and it is believed to provide about a 70% protection rate over the lifetime of the animal given this vaccine (21,42). However, studies have shown that administering a booster shot to calves may afford added protection (38). Strain 19 elicits a mainly CMI response which is very important in brucellosis immunity. One drawback of the vaccine, however, is the smooth phenotype of the strain. A smooth strain expresses LPS on the cell surface and induces humoral responses against the O-side chain. Antibodies produced against the O-side chain of the LPS interfere with standard serologic tests. Vaccinated positive reactors cannot be distinguished from infected positive reactors (34,42). Vaccination with strain 19 can induce abortion in pregnant cattle (33). In addition to induction of abortion and interference with serologic tests, strain 19 is also pathogenic to humans (34,39).

While strain 19 apparently provides long term efficacious immunity against infection by *Brucella*, the adverse characteristics associated with the vaccine and its ability to only protect against *B. abortus* species signals a need for an improved vaccine (13). An improved brucellosis vaccine would have the following characteristics: inability to induce O-side chain antibodies that interfere with serologic tests, induction of long-term effective immunity with one dose, and inability to cause abortion or induce infection in vaccinates and humans. The vaccine should also be a stable strain that does not revert to virulence *in vivo* (45).

The vaccine strain *B. abortus* RB51 meets these criteria; it is a stable rough mutant of *B. abortus* derived from parental strain 2308 (46). The mutant was obtained after passage of 2308 on media containing the antibiotic rifampin (42,45). Following serial passages, a highly attenuated mutant, rifampin resistant and essentially devoid of the O-side chain of LPS, was obtained (46). Strain RB51 passaged through and isolated from mice retains its highly attenuated, avirulent characteristics (13,45,46). Due to the lack of O-side chain in the LPS of strain RB51, vaccination with this strain does not induce antibodies that

interfere with serologic testing of animals. Therefore, it is much easier to distinguish those animals that have been immunized from those which are infected (7,45,46).

Immunity induced by strain RB51 consists of both a humoral and CMI type (45). The CMI response, extremely important to immunity against *Brucella*, seems to be highly induced with this vaccine. One injection confers protection from challenge with strain 2308 and field strains and with an efficacy at least equal to strain 19 (59,70). In addition to providing protection against *B. abortus* strains, a study using a mouse model indicated strain RB51 may be efficacious against *B. melitensis* and *B. ovis* as well (13,70).

In addition to conferring protection against various species of *Brucella*, strain RB51 has other positive characteristics. One very important feature is the apparent inability or very low ability to induce abortion in pregnant animals (7,33,34,45). Also, accidental exposure to the strain during vaccination of animals or other situations has not caused disease in humans. This suggests that strain RB51 may be avirulent in humans (13). Numerous studies using strain RB51 as a vaccine have shown that this strain has most, if not all, of the characteristics desired in the ideal *Brucella* vaccine. Due to this, strain RB51 was approved for use against bovine brucellosis in the U.S.A. by the USDA in 1996 (12,13,42).

Prevention of brucellosis in humans is not mediated by vaccination, as it is in animals, but by eradication of the disease in natural hosts (12,38). In the past, vaccination in humans against brucellosis was used to prevent the disease. Varied successes and adverse effects accompanied use of these vaccines.

Strain *B. melitensis* Rev 1, the live vaccine strain used to immunize sheep and goats, has been studied for efficacy in humans and primates. Results showed that finding a dose, which was protective but not cause disease, was too difficult to justify using strain Rev 1 in humans (38). *B. abortus* 19BA is used in the former USSR to immunize humans against *B. melitensis*; protection lasts for up to one year. The theory behind the use of this vaccine is the idea the *B. abortus* is less pathogenic in humans and can confer cross-immunity to *B.*

melitensis (12,38). Due to the presence of O-side chain on the LPS of strain 19BA, many immunized humans test positive for O-antibodies in serologic testing (38). In China, humans are vaccinated with live attenuated strain *B. abortus* 104M; protection is observed for one year (12,38). Immunization may result in erythema at site of inoculation; other side effects include headache and weakness.

Two non-living vaccines have been used to effectively immunize humans against brucellosis. In the former USSR, a protein-polysaccharide complex derived from the cell wall of smooth *Brucella* strains has been used as an alternative to strain 19BA (12,38). This vaccine is reported as safe and of low reactogenicity when compared to strain 19BA known to cause severe adverse reactions (38). An immunogenic, phenol-insoluble fraction of *B. abortus* and *B. melitensis* has been used to immunize humans in France (12,38). The efficacy of this and other vaccines used in humans have not been well established, but seem to be of limited efficacy (12,38). It is important to notice that the use of all these vaccines has been discontinued due to low protection and side effects.

Various heterologous proteins have been expressed in strain RB51 in an attempt to create a bivalent vaccine. These proteins include, but are not limited to, β -galactosidase (*lacZ*) of *E. coli*. Protein expression has been verified by use of western blot. The recombinant strain induced production of specific antibodies in mice. The LacZ, immunized mice progressed on to challenge by *B. abortus* 2308. Protection against the challenge strain was observed.

Since heterologous gene expression is possible in strain RB51 and mice immunized with the recombinants respond by producing specific antibody, the presence of PA antibodies is expected in mice immunized with strain RB51/PA. The presence of antibodies against PA and the CMI response induced by strain RB51/PA should be sufficient to confer protection against two corresponding bacterial disease threats. The creation and use of this bivalent vaccine could potentially be economically and militarily important.

It is the objective of this research to determine if the *pag* gene encoding the PA antigen of *B. anthracis* can be expressed in *B. abortus* RB51, if the A/J mouse model immunized with the bivalent live vaccine produce specific antibodies against PA, and if immunized A/J mice are protected against a challenge by either virulent *B. abortus* 2308 or avirulent Sterne spore vaccine.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions:

The bacterial strains used in this study are listed in Table 1. *E. coli* DH5 α were grown in Luria-Bertani media (Fisher Biotech, Norcross, GA) or Trypticase Soy Broth (Difco Laboratories, Detroit, MI) (15). *B. abortus* RB51 was grown on Trypticase Soy Broth and SOC-B (6% trypticase soy both, 10mM NaCl, 2.5mM KCL, 10mM MgCl₂, 10mM MgSO₄ and 20mM glucose) (35). All cultures were grown at 37° with shaking. Cultures transformed with plasmids (listed in Table 2) were grown on selective media containing one of the following antibiotics: ampicillin (amp) 100ug/mL, chloramphenicol (Cm) 30ug/mL. Bacto-Agar was purchased from Fisher Biotech (Norcross, GA).

Reagents and enzymes:

Restriction endonucleases and T4 DNA ligase were purchased from Promega (Madison, WI). Ready-to-Go PCR beads were purchased from Pharmacia Biotech (Piscataway, NJ). DNA miniprep kit, PCR cleanup kit, and gel extraction kits were purchased from (Qiagen, Valencia, CA). Agarose was purchased from Fisher Biotech (Norcross, GA). A TA cloning kit was purchased from Invitrogen (Carlsbad, CA). All other reagents were purchased from Sigma Chemical Corporation (St. Louis, MO).

Restriction Digests:

Restriction digests were done in final volumes of 20 or 30ul for single or double digests respectively. Each restriction digest was comprised of 500-1000ng of DNA, 2-3ul of 10x restriction enzyme buffer, 2-12U of restriction enzyme, and distilled water to a final volume of 20-30 uL. Digests were incubated at 37° overnight.

DNA Electrophoresis:

The mini Sub and wide Sub DNA electrophoresis cells (Bio-Rad Laboratories, Hercules, CA) and the power supply EC-100 (E-C Apparatus Corporation, Petersburg, FL) were used to separate DNA fragments in 1% agarose/TBE gels (17.8mM Tris, 17.8mM Boric acid, 0.4mM EDTA); the electrophoresis was conducted at 94V.

NAME	DESCRIPTION	SOURCE
<i>E. coli</i> DH5 α	F ⁻ , ϕ 80dlacZ Δ M15, <i>recA1</i> , <i>endA1</i> , <i>deoR</i> , <i>hsdR17</i> , <i>phoA</i> , <i>supE44</i> , <i>thi-1</i> , <i>gyr A96</i> , <i>Rel A1</i>	BRL ^a
<i>B. abortus</i> RB51	Mutant of <i>B. abortus</i> 2308, lack LPS-O chain (rough phenotype), rifampin R	G.G. Schurig
<i>B. abortus</i> 2308	Wild type virulent field strain, pathogenic in cattle, smooth strain.	G.G. Schurig
<i>B. anthracis</i> Sterne	Unencapsulated, nonpathogenic, toxigenic, spore-forming strain	Colorado Serum Co. ^b

A. GIBCO-BRL Life Technologies, Inc., Grand Island, NY.

B. Colorado Serum Co., Denver, C

NAME	DESCRIPTION	SOURCE
pUTE41	2.96kb <i>HpaI</i> - <i>BamHI</i> pX01 fragment, Containing <i>pag</i> , cloned into pBSIIKS, ampicillin R	T. Koehler ^a
pCR2.1	High copy number plasmid, <i>lacZ</i> +, kanamycin and ampicillin R	Invitrogen ^b
pBBSOD	Partial gene sequence of SOD including promoter added to pBBR1MCS, chloramphenicol R	G.G. Schurig
pBBGroE	Partial gene sequence of GroEL including promoter added to pBBR1MCS, chloramphenicol R	G.G. Schurig
pBBSOD-PA	2.32kb <i>BamHI</i> - <i>XbaI</i> <i>pag</i> gene cloned in pBBSOD, chloramphenicol R	This study
pBBGroE-PA	2.32kb <i>BamHI</i> - <i>XbaI</i> <i>pag</i> gene cloned pBBGroE, chloramphenicol R	This study

A. Dr. T. Koehler, University of Texas, Houston, TX.

B. Invitrogen, Carlsbad, CA.

Plasmid DNA isolation:

DNA plasmids were extracted using the Qiaprep Spin Miniprep kit (Qiagen, Valencia, CA) as per the protocol given by the manufacturer.

PCR amplification of pag gene:

Plasmid pUTE41, obtained from Dr. T. Koehler (University of Texas) contains the complete *pag* gene encoding the protein PA (31). Primers were designed to amplify only the open reading frame (ORF) for the active protein. The forward primer: GGA TCC ACA AAA AGG AGA ACG TAT ATG AAA AAA CGA AAA GTG added a recognition site for the restriction enzyme *Bam*HI. The reverse primer: TCT AGA CAC CTA GAA TTA CCT TAT CCT ATC TCA TAG CCT TTT added a recognition site for the restriction enzyme *Xba*I. The PCR reaction mixture was in a final volume of 25uL and contained primers at a concentration of 1pmol/uL, DNA at a concentration of 50-100ng/uL, and water. The mixture was over-laid by mineral oil to prevent evaporation. The PCR protocol required 35 cycles for completion: denaturation at 65°C for 90 seconds, annealing at 58°C for 90 seconds, and elongation at 72° for 2.5 minutes.

Construction of TA clones:

PCR products were ligated into pCR2.1 of the TA Cloning kit (Invitrogen, Carlsbad, CA) as an intermediate step between PCR amplification of the *pag* gene and construction of the pBBSOD-PA and pBBGroE-PA plasmids. Ligation reactions had a final volume of 10uL and contained a 1:1 to 1:2 ratio of vector to insert, 1uL of 10x ligation buffer, 1U of T4 DNA ligase and water. Ligation reactions were incubated overnight at 4°C and then used to transform *E. coli* DH5 ∞ cells by a heat shock method (16). Transformed cells were plated onto LB-amp plates and grown overnight at 37°C. Colonies were picked from the plate and used to inoculate 10mL cultures of LB broth and grown at 37°C overnight on a shaker. These cultures were then centrifuged at 2060 x g at 4°C to form a pellet. Extraction of DNA plasmid was done using the Qiaprep Spin Miniprep kit from Qiagen. Plasmid DNA was then digested with *Bam*HI and *Xba*I to check for the PCR insert. Plasmid

containing the *pag* gene was used in creating the pBBSOD-PA and pBBGroE-PA plasmids.

Heat shock transformation:

Transformation of *E. coli* DH5 α cells was accomplished using the heat shock method as described by Promega (16).

Construction of the pBBSOD-PA and pBBGroE-PA plasmids:

The broad host range plasmid pBBR1MCS was used to create the pBBSOD and pBBGroE plasmids. A partial sequence of the *Brucella* gene including the promoter region of either superoxide dismutase (SOD) or heat shock protein (GroE) was added to pBBR1MCS to create a plasmid (R. Vemulapalli, VPI&SU).

pCR2.1 containing the 2.32kb *pag* gene was digested using a 16-18 hours digestion with *Bam*HI and *Xba*I. The plasmids pBBSOD and pBBGroE were also digested 16-18 hours with *Bam*HI and *Xba*I. The DNA fragments from these digests were separated by agarose gel electrophoresis and the bands of interest were cut out of the gel. DNA was extracted from these gel slices using the Qiaquick Gel Extraction kit (Qiagen) as per the manufacturer's protocol.

Ligation reactions were set up using the purified DNA. Each ligation reaction had a final volume of 10 μ L and was composed of a 1:2 or 1:3 vector to insert ratio of DNA, 1 μ L of 10x ligase buffer, water and 1U of T4 DNA ligase. These reactions were then incubated at 4 $^{\circ}$ C overnight. *E. coli* DH5 α cells were transformed with these ligation reactions by heat shock method. After transformation, the cells were spread on TSB-Cm plates and incubated overnight at 37 $^{\circ}$ C.

Several clones were picked from those that grew overnight and were checked for recombination. Colonies were used to inoculate LB-Cm 10mL cultures and grown overnight at 37 $^{\circ}$ C with shaking. These cultures were spun down to a pellet at 1800 x g and 4 $^{\circ}$ C. The pellets were then used to extract plasmid DNA using the Qiaprep Spin Miniprep kit (Qiagen).

Extracted plasmid DNA was then digested overnight using *Bam*HI, *Xba*I and *Pst*I at 37°C to check for the *pag* insert and its orientation in the plasmid. The restriction fragments were then separated by electrophoresis in agarose. Those clones, containing the gene of interest in the correct orientation, were used for the rest of this study.

Transformation of *B. abortus* RB51:

Competent *B. abortus* RB51 cells were made and transformed with the plasmid constructs via electroporation (35). Transformed cells were plated onto TSB-Cm plates and incubated at 37°C for 4 days. Cm resistant colonies were tested for transformation by Western blot using specific PA antiserum.

SDS-PAGE of clones:

Extracts or media of *E. coli* DH5 α and *B. abortus* RB51 transformed with the plasmid pBBSOD, pBBGroE, pBBSOD-PA, or pBBGroE-PA were separated on SDS-PAGE gels to check for expression of PA. To check for this expression, sample aliquots from both cell pellets and culture media were examined on gels. Laemmli described the procedure followed with some modifications (32). A resolving gel: 12.5% acrylamide (Bio-Rad), 10% SDS, 3M Tris-HCl, pH 8.8, 0.3% TEMED, 1.5% ammonium persulfate. A stacking gel: 4% acrylamide, 10% SDS, 0.5M Tris-HCl, pH 6.8, 0.3% TEMED, 1.5% ammonium persulfate. HPLC-grade water was also used in both the resolving and stacking gel.

Western blot:

Gels were run for 90 minutes at 25mA per gel. Proteins were then transferred from the gel to nitrocellulose membrane (7.5cm x 10cm) in preparation for immunoblotting by electro-transfer. Nitrocellulose membranes with attached proteins were blocked in 1% bovine serum albumin for 3 hours to prevent non-specific binding of the 1° antibody to the membrane. The blocked membranes were then exposed to the 1° antibody at a 1:1000 overnight on a shaker at 4°C. The membranes were then washed three times and 2° antibody at a 1:2000 dilution added to the blot for 2-3 hours at room temperature. The

membranes were washed again three times and the substrate, 4-chloro-1-naphthol, was added to the blot. Purple color developed where the primary antibody recognized protein bands.

Immunization of mice with *B. abortus* RB51/pBBGroE±PA:

Twenty-six A/J mice (Jackson Laboratories, Bar Harbor, ME) were divided into 3 groups (Table 3). Five mice were designated as controls and injected intraperitoneally (IP) with 0.2mL of sterile saline. Ten mice were designated the GroE group and were injected IP with 3.6×10^8 cfu of *B. abortus* RB51 transformed with pBBGroE plasmid. Eleven mice were designated the pBBGroE-PA group. These mice were injected IP with 4.3×10^8 cfu of *B. abortus* RB51 transformed with the pBBGroE-PA plasmid.

Challenge of mice:

The mice were bled at 6 and 8 weeks post immunization. At 8 weeks post immunization, the mice were challenged. Three naïve mice and 5 mice from each of the pBBGroE and pBBGroE-PA groups were injected IP with 2.4×10^4 cfu of *B. abortus* 2308. At 2 weeks post challenge the mice were sacrificed and the spleens were harvested, homogenized and aliquots plated on TSB plates to determine the clearance of the strain 2308.

The 5 mice receiving saline, 5 pBBGroE mice as well as the 6 pBBGroE-PA immunized mice were then injected IP with 5.6×10^4 spores of *B. anthracis* Sterne strain.

Western blots using mouse serum:

Pure PA protein (~3ug) was loaded into each well of an SDS-PAGE gel, electrophoresed and transferred to nitrocellulose membranes as mentioned above. Membranes were blocked with 1% BSA and cut into strips; each strip represents one lane of PA. Each strip was placed into a sealed bag and 1 mL of mouse sera of a 1:25 dilution in 1% BSA was added. These strips were incubated at 4°C for 3 days. The strips were

EXPERIMENTAL DESIGN

GROUP	N	VACCINE	CHALLENGE ORGANISM	N
1	8	SALINE	<i>B. abortus</i> 2308	3
			<i>B. anthracis</i> Sterne spores	5
2	10	RB51	<i>B. abortus</i> 2308	5
			<i>B. anthracis</i> Sterne spores	5
3	11	RB51/PA	<i>B. abortus</i> 2308	5
			<i>B. anthracis</i> Sterne spores	6

Table 3

then washed 3 times with 10mL of TBS and exposed to 2° antibody at a 1:1000 dilution for 3 hours. Addition of the 4-chloro-1-naphthol substrate after washing revealed which mice were producing antibodies to PA by the presence or absence of a band corresponding to the site where full size PA would be located on the membrane.

Statistical Analysis

Counts of bacterial cfu in the spleens of mice were analyzed by the paired *t* test using Sigma Plot software. P values of >0.01 were considered significant for this study.

RESULTS

Research flow chart:

The flow chart outlining the research for this thesis is presented in Figure 3.

Construction of pCR2.1-PA plasmid:

This plasmid is used as an intermediate step between PCR amplification of a gene and ligation into the intended plasmid in order to make sure the insert had the desired size, restriction enzyme recognition sites and orientation.

The plasmid pUTE41, containing the intact *pag* gene encoding for PA was used as our PCR template. Primers were designed (see Materials and Methods) to amplify the longest ORF (2.32kb) from the *pag* gene in order to obtain full size protein. PCR amplified products were run on 1% agarose gels; bands were excised and cleaned (Figure 4). Ligation of the *pag* gene insert into pCR2.1 and subsequent transformation of *E. coli* DH5 α yielded amp resistant colonies. The pCR2.1-PA plasmid was purified and digested.

Construction of pBBSOD-PA and pBBGroE-PA:

Digestion of pCR2.1-PA with *BamHI* and *XbaI* dropped out the insert gene. Digestion of both pBBSOD and pBBGroE with *BamHI* and *XbaI* allowed subsequent ligation of the 3.23kb insert into each plasmid (Figure 5, 6). Transformation of *E. coli* DH5 α cells yielded Cm resistant clones. Restriction digests of the plasmid purified from these clones showed the *pag* insert in proper orientation. Single and double digests were performed on each plasmid with *BamHI* and *XbaI* in order to determine the presence of the *pag* gene. Each double digest of a recombinant plasmid shows a DNA fragment band at the desired 2.32kb (Figure 7).

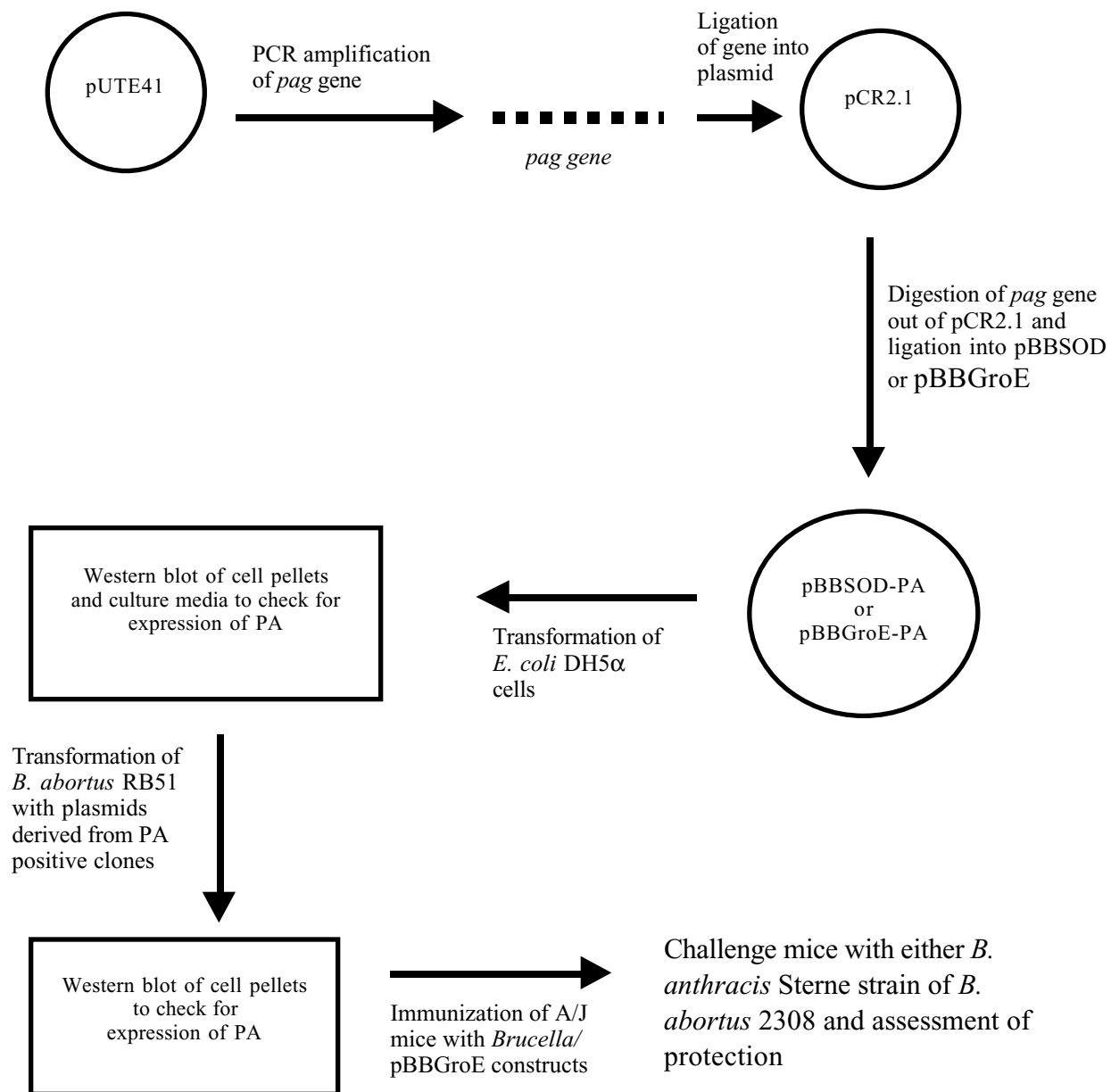


Figure 3: Flow chart for the research conducted in this thesis research.

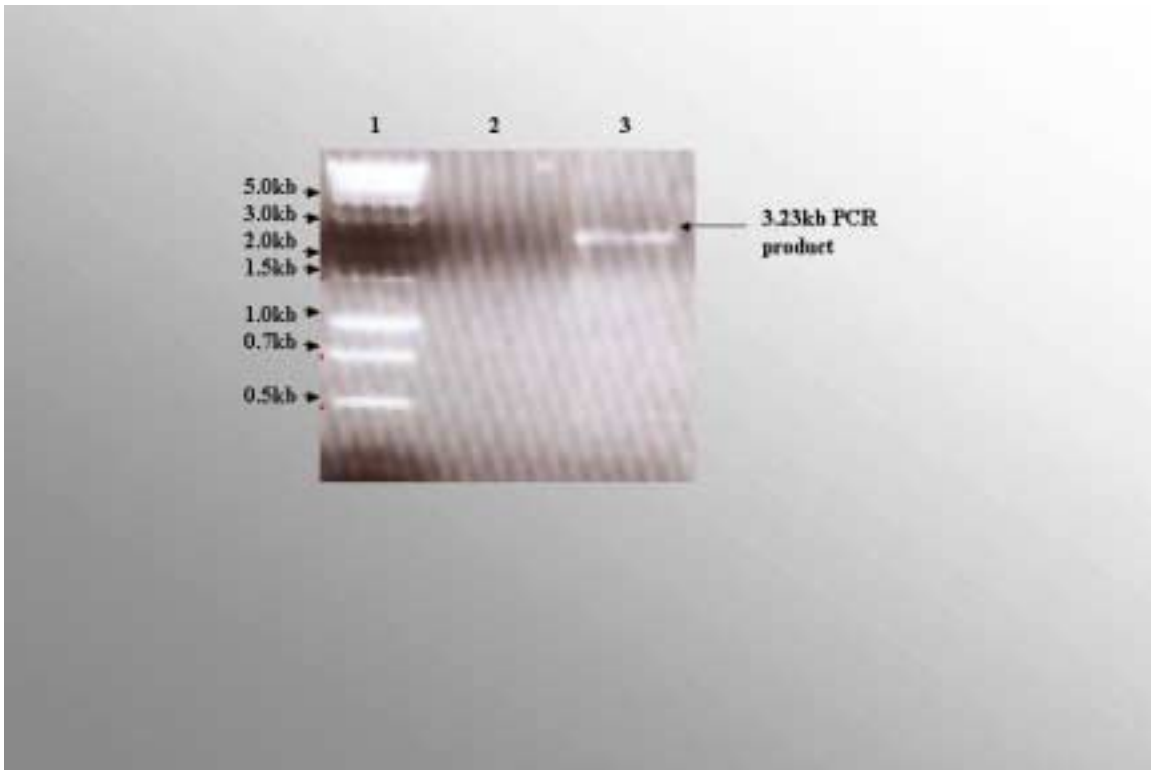


Figure 4: Agarose gel of PCR fragments derived from pUTE41. Lane 1, is a DNA marker ladder; lane 2, no template; lane 3, *pag* template.

Expression of PA in *E. coli*:

Recombinant clones were cultured in preparation for a Western blot. Since PA is a secreted protein when synthesized by *B. anthracis*, the culture media as well as the *E. coli* cells were checked for the presence of the protein (53). Preliminary blots indicated that the PA protein was very susceptible to degradation from proteases in the *E. coli* and from heat as reported (487). In order to alleviate heat degradation, the cell pellets and culture media were harvested at 4°C. Although full size PA protein was not able to be visualized on a Western blot, several bands corresponding to degradation products of PA were identified (40). These bands were not only seen in the cell pellets but also in the culture media. These observations suggest the *E. coli* is able to fold and package PA to its secretory pathway (Figure 8).

Expression of PA in *B. abortus* RB51:

The pBBSOD-PA or pBBGroE-PA plasmids were used to transform *B. abortus* RB51 to create a bivalent vaccine against brucellosis and anthrax. Competent *B. abortus* RB51 cells were transformed by electroporation and plated onto TSB-Cm plates (35). Transformed cells resulted in Cm resistant clones; cultures were grown of each clone for use in Western blot to check for expression of PA.

Before the *Brucella* clones could be removed from the BL-3 lab, they were killed by heating the cultures to 64°C for 1 hour. The cultures were placed on ice while being prepared for SDS-PAGE electrophoresis. Cell pellets and culture media were collected as samples solubilized in loading buffer and separated by SDS-PAGE (32). Full size PA was not visualized on these gels. However, the degradation product bands seen in the blots of *E. coli* cell pellets were present in *Brucella* as well. This degradation of PA in *Brucella* could have resulted during the heat inactivation of the RB51 cells (Figure 9).

Stabilization of *B. abortus* RB51 cultures for Western blot:

Brucella cultures were kept on ice for every step of sample preparation, except for the 1 hour heat inactivation step. While this treatment helped stabilize full-length PA (63-

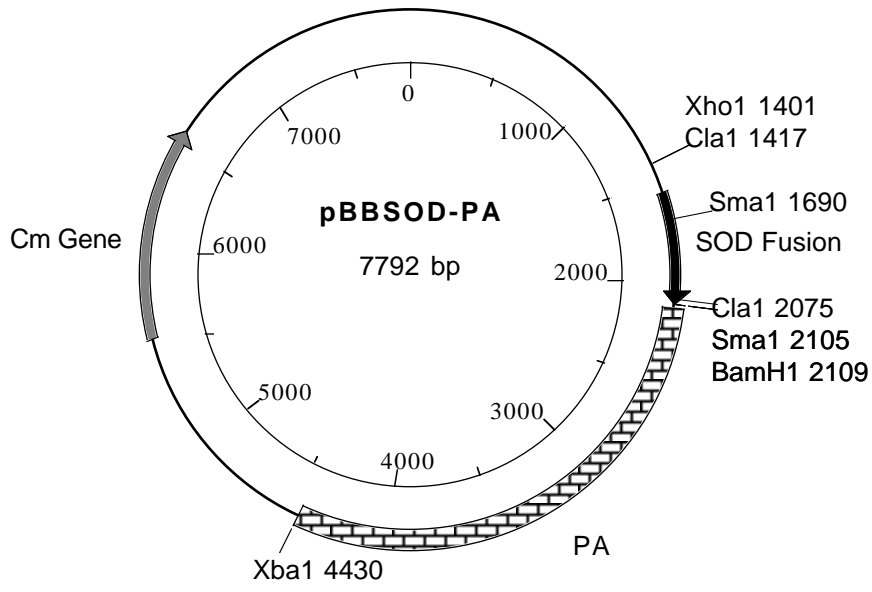


Figure 5: Plasmid pBBSOD-PA. Derived by ligation of 3.23kb *pag* gene encoding PA into *BamHI* and *XbaI* sites of pBBSOD (R. Vemulapalli, VPI&SU).

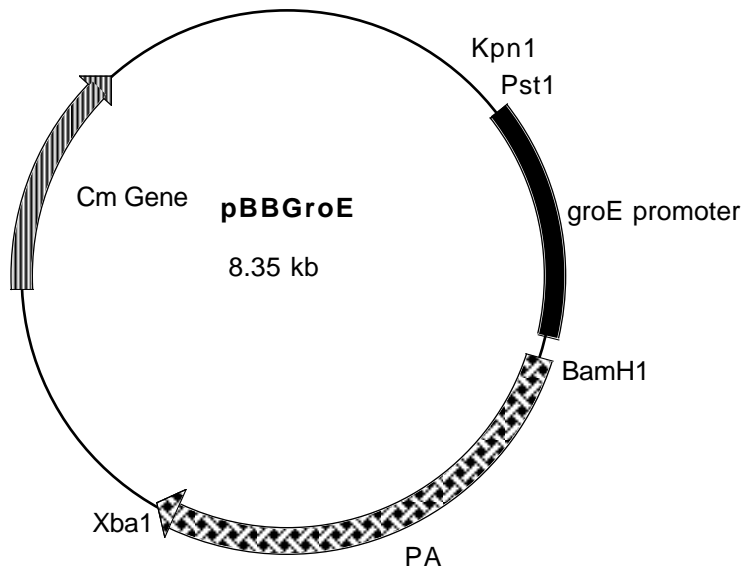


Figure 6: Plasmid pBBGroE-PA. Derived by ligation of 3.23kb *pag* gene encoding PA into *BamHI* and *XbaI* sites of pBBGroE (R. Vemulapalli, VPI&SU).

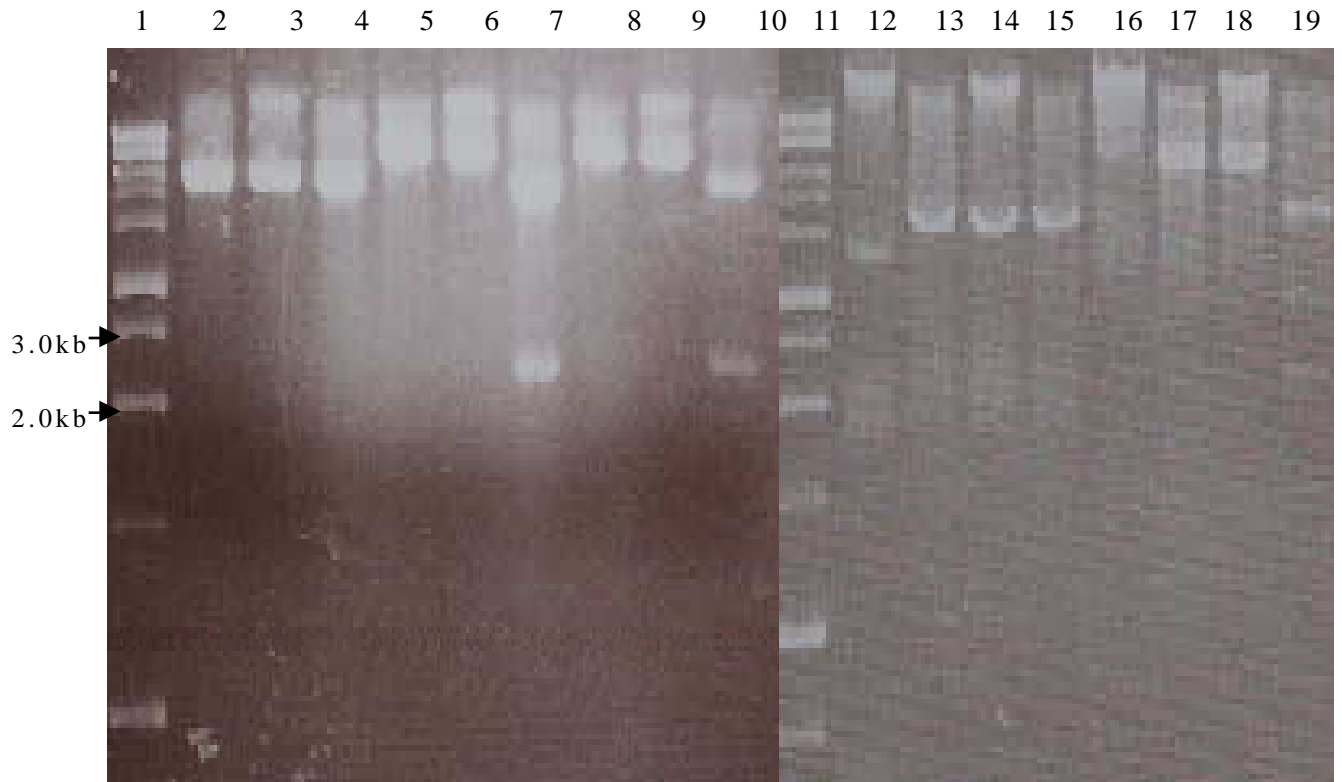


Figure 7: Restriction digests of plasmids pBBSOD and pBBGroE which contain the *pag* gene using *Bam*HI and *Xba*I. Lanes 1 and 11 are DNA marker ladders . Lane 2 and 3 are single digests of pBBGroE, lane 4 is a double digest of pBBGroE. Lane 5, 6, 8 and 9 are single digests of pBBGroE-PA, lane 7 and 10 are double digests. Lane 12 is undigested pBBSOD, lane 13 and 14 are single digests, and lane 15 is a double digest. Lane 16 is uncut pBBSOD-PA, lane 17 and 18 are single digests, lane 19 is a double digest.

83kDa), the color intensity of the band was very faint and could not be photographed. However, this blot gave proof that *B. abortus* RB51 was able to produce full size PA protein.

To attempt to further enhance the expression of PA in strain RB51, the heat inactivation step was left out of the sample preparation process. Cultures were kept on ice and the inactivation step was the addition of Laemmli sample buffer to cell pellets and vortexing; this buffer lyses the cells. The omission of the heat inactivation step seemed to significantly reduce PA degradation in *Brucella*. When the proteins from cultures were run by SDS-PAGE gel, a dark band corresponding to full size protein could be seen (Figure 10, 11). However, only a dark band in the lanes corresponding to the pBBGroE-PA construct was observed. A faint band corresponding to the full size protein was seen in the pBBSOD-PA protein. Thus it is possible that the GroE promoter is better at expressing synthesis of PA in *Brucella* than the SOD promoter.

No bands were seen in any of the lanes containing the *Brucella* culture media samples.

Immunization of BALB/c mice with strain RB51/pBBSOD-PA:

Since the pBBSOD-PA plasmid was constructed first, it was tested in mice before the pBBGroE-PA construct. Six female BALB/C mice (Charles River Laboratories, Wilmington, MA) were used for this pilot study. Three mice were placed in each group and injected IP with *B. abortus* RB51 containing either the pBBSOD or pBBSOD-PA construct at a dose of $\sim 5 \times 10^8$ cfu. Four weeks later the mice were bled, and Western blots showed no reaction of the mouse sera to pure PA. The mice were bled 8 weeks after immunization to check for the presence of antibodies against PA; again Western blots showed no reaction. Each mouse was given a second immunization of $\sim 2.5 \times 10^8$ cfu *B. abortus* strain RB51/SOD-PA and were bled 2 weeks later; no antibodies against PA were detected. Because other studies showed the SOD gene is down regulated in *Brucella in*

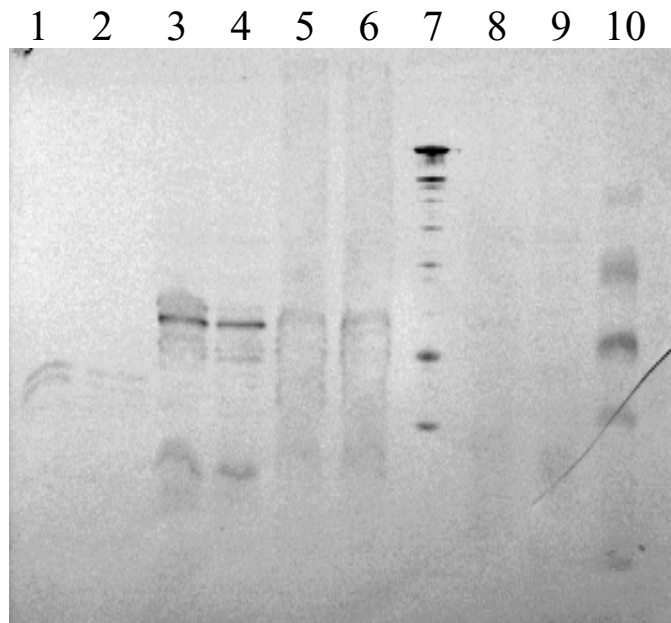


Figure 8: Immunoblot of *E. coli* expressing PA. Lanes 1 and 2 are culture media showing bands of PA degradation products. Lanes 3, 4, 5 and 6 are cell pellets of *E. coli*/pBBSOD-PA showing bands of degradation products of PA. Lane 7 is purified PA protein (~3ug). Lanes 8 and 9 are cell pellets of *E. coli*/pBBSOD. Lane 10 is the protein molecular mass marker.

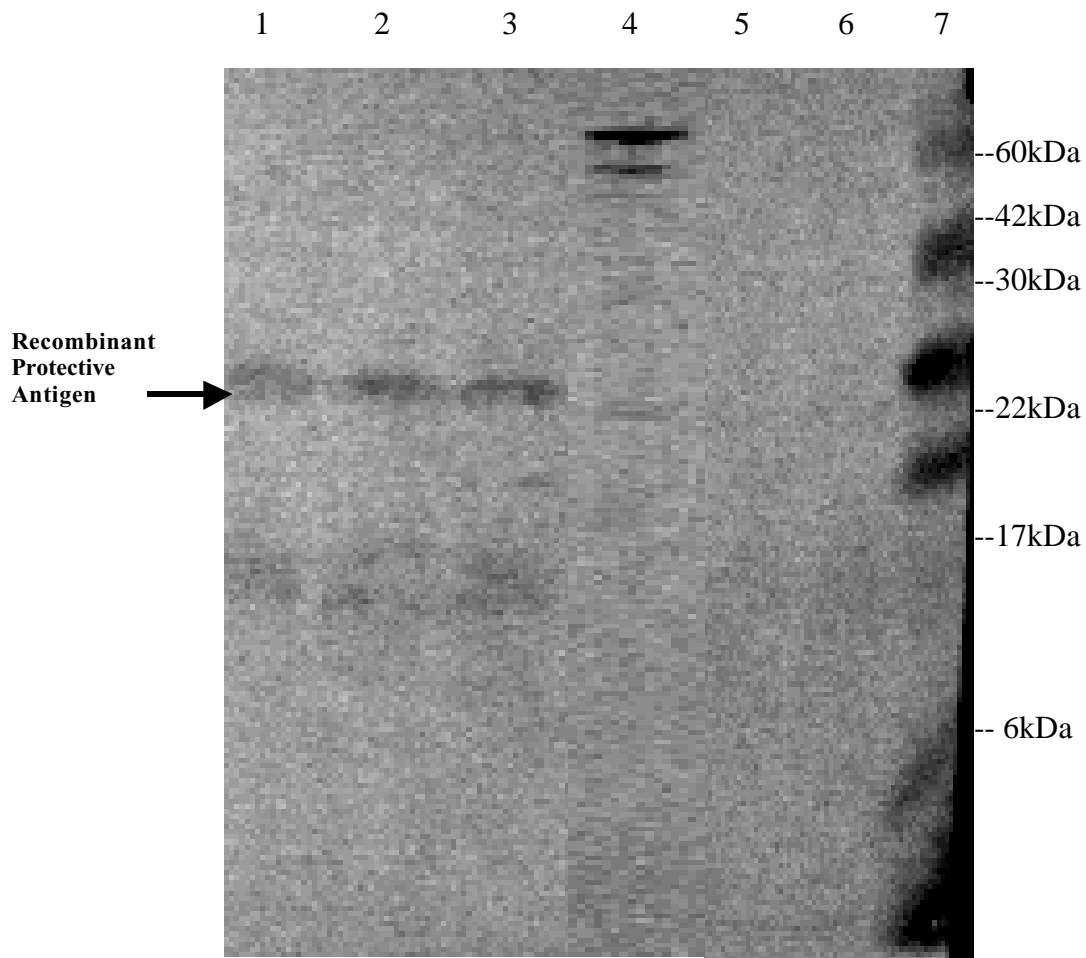


Figure 9: Western blot of *Brucella* clones expressing PA. The bands corresponding to ~27kDa represent degradation products of PA. Lanes 1-3 are cell pellets of *Brucella*/pBBSOD-PA clones, lane 4 is purified PA protein (~3ug), lanes 5-6 are *Brucella*/pBBSOD cell pellets, and lane 7 protein molecular mass markers.

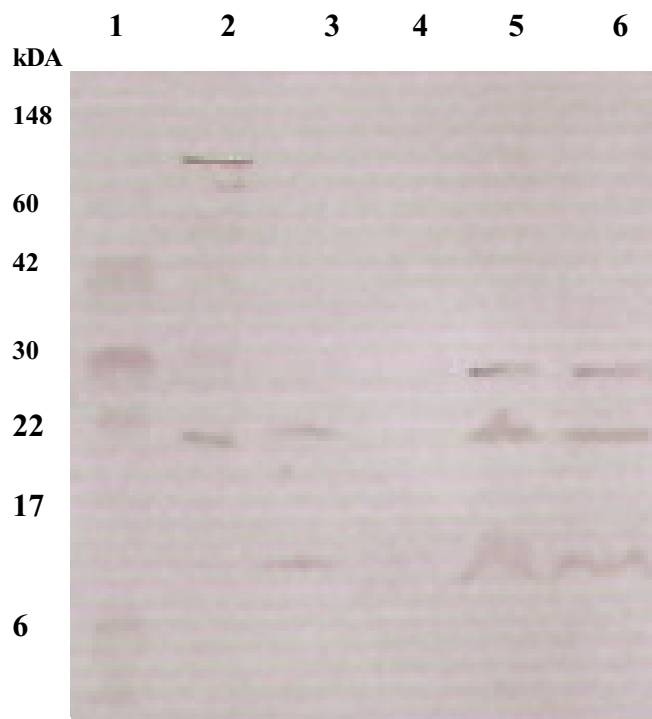


Figure 10: Western blot of *Brucella*/pBBSOD-PA killed by sample buffer. Cells were harvested under cold conditions and killed by addition of Laemmli sample buffer. Lane 1 is the marker, lane 2 is pure PA protein, lanes 3-4 are cells from *Brucella*/pBBSOD, and lanes 5-6 are cells from *Brucella*/pBBSOD-PA. There is a very faint band in lane 6 which corresponds to PA of ~63kDa, however, it was too faint to photograph.

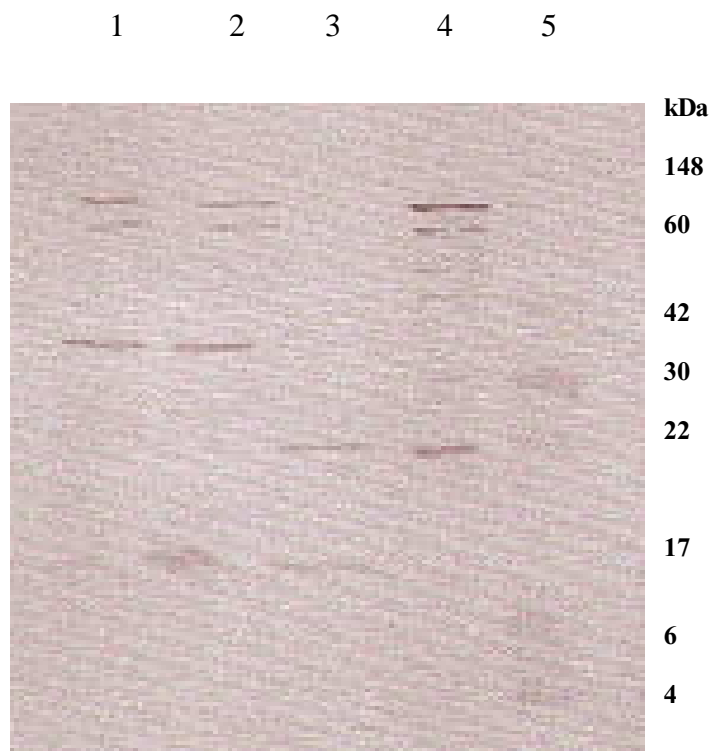


Figure 11: Western blot of *Brucella*/pBBGroE-PA clones. Samples were harvested under cold conditions and cell extracts prepared by adding Laemmli sample buffer. Lanes 1-2 are pBBGroE-PA clone cell pellets, lane 3 is pBBGroE cell pellet, lane 4 is pure PA protein and lane 5 is protein molecular mass markers.

vivo (unpublished data, G. Schurig, VPI&SU), it was decided to use the pBBGroE-PA construct in the vaccine trial. Since GroE is a heat shock protein, it is assumed to be up regulated during times of stress such as when *Brucella* is replicating in macrophages.

Protection assessment of vaccine trial with strain RB51/pBBGroE-PA:

The mice in this trial were separated into groups by designation of the immunizing construct strain RB51: pBBGroE, pBBGroE-PA or sterile saline, and by the challenge organisms, *B. abortus* 2308 or *B. anthracis* Sterne strain.

However, in an attempt to create a safer environment for the researchers, A/J mice, a mouse model strain susceptible to avirulent anthrax spores, was used for this protection study (67,68). Therefore, we could use the avirulent spores of the live spore vaccine as a challenge, rather than the more dangerous virulent field strains normally used in protection studies (68).

Twenty-six female A/J mice were divided into 3 groups. Five mice were designated controls to be injected with sterile saline, and 10 mice were designated to be injected with *B. abortus* RB51/pBBGroE. The remaining 11 mice were to receive *B. abortus* RB51/pBBGroE-PA. The mice were bled at weeks 6 and 8 after immunization to provide sera to assess humoral antibodies. Western blot revealed the presence of antibodies against PA in the serum at week 6; antibodies persisted through to week 8 in mice immunized with strain RB51/pBBGroE-PA. Of the eleven mice injected with the pBBGroE-PA construct, 10 were positive PA reactors (Figure 12).

Since strain RB51 is able to confer protection against *Brucella* after a single immunization, and antibodies against PA were present to protect against anthrax, these animals were challenged. At 56 days post-immunization, the five saline mice, five of the mice receiving strain RB51/pBBGroE and six mice receiving strain RB51/pBBGroE-PA were challenged with 5.6×10^4 spores of the Sterne strain. The remaining mice were challenged with 2.4×10^4 cfu's of *B. abortus* 2308.

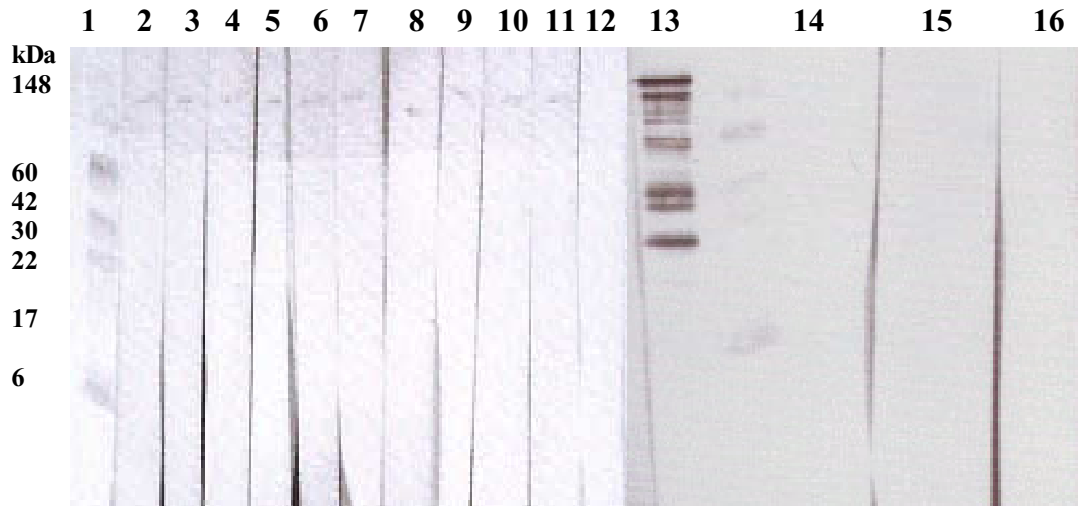


Figure 12: Western blots of pure PA protein exposed to sera from immunized mice. Lanes 1 and 14 are protein molecular mass markers. Lanes 2-12 are pure PA (~3ug) exposed to sera from mice immunized with *Brucella* expressing PA (pBBGroE-PA). Lane 13 is pure PA protein (~3ug) exposed to rabbit anti-PA serum. Lane 15 is pure PA (~3ug) exposed to serum from a mouse immunized with sterile saline, lane 16 the PA protein was incubated in serum from a mouse immunized with *Brucella* not expressing PA (pBBGroE).

The endpoint for those mice challenged with Sterne strain was death; any survival indicates protection (Figure 13). The saline group survived for 4 days before all mice in the group died. The group immunized with strain RB51/pBBGroE survived longer than the saline group but all mice in the group eventually died by day 7. The mice receiving strain RB51/pBBGroE-PA survived even longer; one mouse was able to survive the challenge dose altogether. This extended survival rate suggests that the antibodies produced by the mice due to the immunization were somewhat protective.

The endpoint for those mice challenged with *B. abortus* strain 2308 was determination of splenic clearance of strain 2308 from the mice. Mice were sacrificed and spleens harvested and cultured to observe clearance of the *Brucella* (Figure 14). In order for a vaccine to be considered protective it must confer at least 1 log of protection over that achieved by saline alone. The clearance data suggests that the additional expression of the PA protein in *B. abortus* strain RB51 does not effect its ability to protect against a *Brucella* challenge. Comparison of groups immunized with either strain RB51/pBBGroE or RB51/pBBGroE-PA showed no difference in the level of protection.

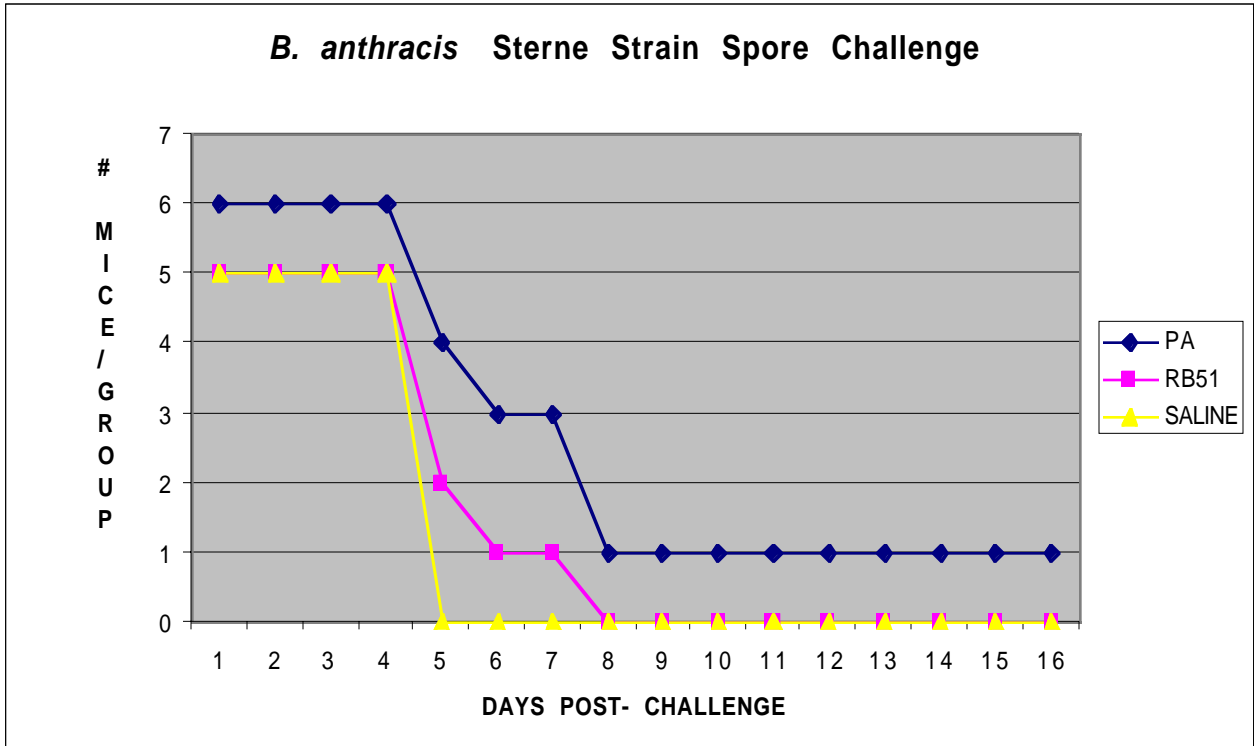


Figure 13: Survival of mice challenged with Sterne vaccine spores. Each mouse at day 0 received 5.6×10^4 spores IP. The PA group corresponds to mice immunized with the pBBGroE-PA construct. The RB51 group corresponds to the mice immunized with the pBBGroE construct. The saline group corresponds to the mice in the control group immunized with sterile saline.

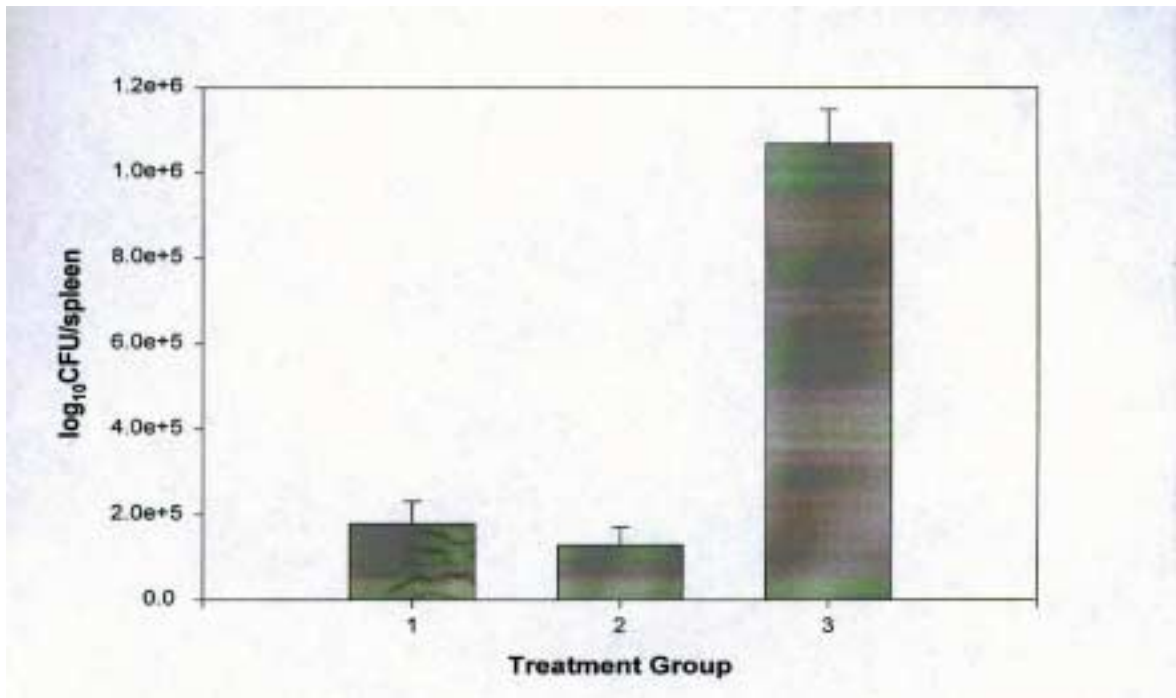


Figure 14: Clearance of *B. abortus* 2308 from A/J mice. Time of challenge was at 56 days post-immunization. Group 1 was immunized with the strain RB51/pBBGroE, group 2 with the strain RB51/pBBGroE-PA, and group 3 with sterile saline. The P value (by the paired t-test) for the t-test of group 1 vs. group 3= 0.000251; group 2 vs. group 3=0.00905. The P value for the t-test of group 1 vs. group 2= 0.403.

DISCUSSION

Brucellosis is virtually eradicated in the domestic cattle population of the continental United States, Canada, and Western Europe. However, the same cannot be said for cattle in developing nations where the disease can be considered endemic. The availability of a successful vaccine against *Brucella*, such as strain RB51, is one tool being used by these countries to eradicate the disease in cattle. Since the vaccine is relatively easy to produce and requires only one dose for effective immunity, it is a cost effective way to battle brucellosis (21).

Nations in which brucellosis is endemic often have the same level of anthrax prevalence (21). While the currently available vaccines against *B. anthracis* are effective, they do have some undesirable side effects including virulence in humans and some animal species (24,38).

Since these two diseases tend to be widespread in developing countries, it is often unaffordable for livestock owners to vaccinate against both anthrax and brucellosis (19,21). A desirable alternative would be to develop a bivalent vaccine that would deliver effective immunity against both diseases using only one or two immunizations. This vaccine would contribute greatly to the eradication of two diseases at one time and allow for economic growth through the ability to trade livestock with disease free nations.

Since strain RB51 is such a successful vaccine, it is a promising choice to serve as a delivery system (13). As stated before, one immunization confers effective protection against *Brucella* infection. This strain has also been used in lab studies to deliver homologous and heterologous proteins for immunization of mice (70).

Proteins, such the *lacZ* protein of *E. coli*, have been expressed in strain RB51. These recombinant strains were then used to immunize BALB/c mice. Western blots showed the presence of antibodies in the mouse sera against these proteins. This is indicative of strain RB51's ability to synthesize recombinant proteins that are antigenic. Challenge of these immunized mice with *B. abortus* 2308 has shown that synthesis of these

heterologous proteins does not interfere with strain RB51's ability to induce protective immunity against virulent strains of *Brucella* (unpublished data, R. Vemulapalli, VPI&SU).

While we know that strain RB51 is protective and able to synthesize heterologous antigens, those proteins need to be potent antigens in order to induce protection. In the case of this study, the PA protein of *B. anthracis* was chosen, since numerous studies have shown PA induces protective antibodies in immunized humans and animals; it is also the most common component of any anthrax vaccine (24,48,60).

The bivalent vaccine developed here used strain RB51 to synthesize and deliver PA in immunized mice. One very important aspect of this bivalent vaccine's characteristics is the ability of the synthesized PA to offer significant but limited protection against challenge by *B. anthracis* spores. Since the induced antibodies to PA are not protective, the vaccine's level of PA expression and immunization protocol needs to be fully reevaluated.

Previous studies using other bacterial strains as delivery systems for PA include *S. typhimurium* and *B. subtilis* and virus vaccinia (14,23,30). The recombinant PA expressed in these systems was effective at providing protection in mice against *B. anthracis* spore infection. *S. typhimurium* expressing PA was used as an oral vaccine in a trial comparing its protective efficacy to adjuvanted PA. Protection studies in mice showed the recombinant vaccine to be as effective as the adjuvanted PA in providing protection with efficacy rates of 33% (14). Recombinant strains of *B. subtilis* expressing PA used as live vaccines have been compared in vaccine trials to the AVA and live spore vaccines. Study results have shown that the *B. subtilis* clones have protective efficacies in mice better than AVA and equal to the live spore vaccine with a protection rate of up to 90% (1,26,30). Vaccine trials to determine the efficacy of vaccinia virus strain WR against anthrax infection have shown a 60% protection rate in mice (23).

In addition to the antibodies induced, the ability of these strains to protect against infection by *B. anthracis* spores could be due to the nonspecific induction of CMI, which has been theorized to be an important component of anthrax immunity (2,27,28,29). Since

CMI has not been eliminated as a protective component of recombinant bacterial strains expressing PA, strain RB51 seems to be an ideal bacterial vector for the creation of a bivalent vaccine. Strain RB51 is a strong inducer of CMI and its ability to synthesize heterologous proteins that induce antibodies in mice indicate that its production of PA could be protective in nature due to the induction of both CMI and antibodies (45,59,70).

Western blots of strain RB51/pBBGroE-PA have revealed the strain's ability to produce full size PA. This is essential in the development of PA antibodies, as PA degradation products are unable to induce antibodies since they are biologically inactive both as antigens and toxin components (40). To further support the theory that the PA synthesized by strain RB51 is immunogenic, 11 female A/J mice were immunized with *B. abortus* strain RB5/ pBBGroE-PA. Of the 11 mice immunized, 10 developed PA antibodies as seen by the western blots.

Mice vaccinated with the bivalent vaccine were challenged by both bacterial strains, *B. abortus* 2308 and *B. anthracis*. The first and most common way to test the efficacy of an anthrax vaccine is to challenge immunized mice with spores from a virulent strain of *B. anthracis*. The second and somewhat safer way is to use a strain of mouse that is susceptible to the veterinary live spore vaccine produced by the Colorado Serum Company. The live spore vaccine is derived from an avirulent strain of *B. anthracis*, known as Sterne, and is toxigenic and non-encapsulated. Injection of a lethal dose of spores into mice such as A/J results in a course of infection resembling the pathogenesis of disease of fully virulent spores (67,68). Injection of $\sim 10^4$ spores results in 80-90% mortality in about 6 days (68). This is comparable to injection of mice with 6 spores from virulent *B. anthracis* Vollum 1B spores resulting in death in about 3 days (67). Therefore, since injection of susceptible mice with an avirulent strain of *B. anthracis* gives similar pathogenesis and endpoint, it could be considered a useful and safer model of challenge when testing new vaccine efficacy (68).

The protection study in this thesis yielded some promising results. Challenge with 2.4×10^4 cfu's of *B. abortus* strain 2308 and subsequent splenic clearance studies revealed that the strain RB51 immunized mice (both the pBBGroE and pBBGroE-PA) had a rate of clearance 1 log greater than the saline immunized group. The strain RB51 vaccine is considered successful in providing protection if it confers protection in terms of splenic clearance at the level of 1-2 logs greater than saline control mice (59). In addition, the groups challenged with *B. anthracis* Sterne strain provided some indications of protection. These mice were given a challenge dose of 5.6×10^4 spores and observed for signs of illness and subsequent death. For unimmunized A/J mice receiving this dose of spores, the expected mortality rate is ~95% with an average time to death of ~4.5 days (68). On days 1-3 post challenge, mice in all groups were observed for signs of illness. On day 2, all mice presented with scruffy coats and reduced activity and appetite. On day 3 all mice were inactive and not eating. On day 4, all mice in the saline control group died as well as 3 mice in the pBBGroE group and 2 in the pBBGroE-PA group. On day 5, one mouse from each of the 2 remaining groups died. By day 7 post challenge, all mice from the saline and pBBGroE groups were dead and 1 mouse from the pBBGroE-PA group survived. By day 8, this surviving mouse's coat returned to its normal appearance and activity and appetite increased.

Overall, the trends of the survival curves of each challenged group are encouraging. The saline group survived the expected period of time before succumbing to the challenge dose. The mice in the pBBGroE group died more slowly than those in the saline group. This would seem to indicate a role of CMI in some non-specific protection of these mice. However, this non-specific protection was not enough to protect these mice as they also died from the challenge. The mice in the pBBGroE-PA group survived the longest before succumbing to the challenge dose including one survivor.

While the overall protection rate is not as impressive as the protection rates of other recombinant bacterial systems producing PA, the level of protection and increased time to

death post challenge indicates that the strain RB51 bivalent vaccine could potentially be a good candidate for vaccination against anthrax and brucellosis. Further studies are needed to determine if strain RB51 expressing PA can be optimized in terms of the protection afforded.

These future studies would include a repeat of the protection trial just described with several variations. One variation would be to give a single immunization as in this study but include 2 groups of mice immunized with the pBBGroE and pBBGroE-PA constructs to be challenged with the Sterne spores at 4 and 8 weeks post immunization. This would allow us to determine if the increased time to death seen in this study may have been due to non-specific CMI induced by strain RB51. Another possible change in the protection study would be to include a second immunization before challenge. Other studies have indicated that more than a single exposure to PA is required to develop protective levels of antibodies (23,25,26,35). The addition of another variable to the protection study could be to immunize the mice with a DNA based vaccine plasmid carrying the *pag* gene after the initial administration of the strain RB51 constructs in the mice. Unpublished data indicate a combination immunization protocol employing both live vaccines and DNA based vaccines boosts immunity significantly when compared to protocols using only one type of vaccine (G. Schurig, VPI&SU). Finally, the development of an ELISA, to assess the titer of PA antibodies in each mouse could be useful in assessing the vaccine's efficacy. While high anti-PA titers do not necessarily correlate to high levels of protection, knowing these titers could be helpful in characterizing strain RB51's ability to synthesize immunogenic and protective PA and the subtypes induced by the vaccine (20,59).

In summary, the results of this thesis research have demonstrated that *B. abortus* RB51 is able to express the full size PA protein of *B. anthracis*. In addition, the immunization of A/J mice and subsequent challenge with *B. abortus* strain 2308 shows no interference in vaccine strain RB51's ability to confer protection while expressing PA. The challenge of A/J mice with *B. anthracis* Sterne strain indicates that the PA produced by

strain RB51/pBBGroE-PA is able to confer some protection. Also, this study has shown that the utilization of the A/J mouse as a model for anthrax and brucellosis is possible even though the mice have defective macrophages (67,68).

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