Brucella infection and vaccine studies in feral and domestic swine

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Brucella infection and vaccine studies in feral and domestic swine

by

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CHAPTER 1: GENERAL INTRODUCTION

Overview

The genus *Brucella* includes eight species which infect numerous mammalian hosts as well as humans. In the natural animal hosts the brucellae infect the lymphoreticular and reproductive organs. These infections often lead to pyogranulomatous lesions in numerous organs. In humans the disease has been historically referred to as undulant fever and is characterized by recurrent pyrexia, malaise, myalgia, abortion in pregnant females, and pyogranulomatous lesions in male reproductive organs, joints, and parenchymatous organs.

The first isolation of *Brucella* was made by David Bruce in 1887 on the island of Malta from the spleens of infected British soldiers and was originally named *Micrococcus melitensis*. Later work on Malta found that the organism which Bruce identified was also found in the milk of goats. The genus was later renamed *Brucella* in honor of Bruce’s contribution to discovering the etiologic agent of undulant fever. The first isolation of *Brucella* in animals, however, was made by Bang in Denmark in 1897. Bang also was the first to fulfill Koch’s postulates by reproducing abortion in pregnant cattle with the organism which he isolated. The first isolation of *Brucella* in swine occurred in 1914 in Indiana and was accomplished by Traum. Traum isolated the organism from an aborted fetus and identified the organism as a unique strain of *Brucella abortus*, which was at that time known as *Bacillus abortus*. The causative agent of swine brucellosis was later recognized and classified as a separate species in the genus *Brucella* by Huddleson in 1929, and was renamed *Brucella suis*.

The first eradication program for brucellosis in the United States was initiated for cattle in 1934. This program was started subsequent to a major drought and the great depression more as an effort to reduce cattle numbers within the country as part of an economic recovery program rather than to control brucellosis. Since its inception, this program has relied on identification of seroreactive animals, identification of herds of origin, and elimination of seroreactive cattle or herds. This program in cattle was aided
by the introduction of vaccination with the *B. abortus* strain 19 in 1940 and *B. abortus* strain RB51 in 1996.

The US State-Federal Brucellosis Eradication Program for swine was organized in the 1960’s. Like its cattle counterpart, this program has relied on serological surveillance, epidemiological investigation, trace back to originating herd, and elimination of seroreactive herds. Vaccination has never been a part of the swine eradication program; however, research into the application of *Brucella* vaccines to swine occurred in the 1940’s and 1950’s prior to the initiation of the program. The success of the program was undoubtedly aided by the drastic changes in swine production and management which have occurred in the industry since the program’s inception. These changes have lead to the institution of biosecurity practices and record keeping which can only be rivaled by the poultry industry.

The successful elimination of swine brucellosis from domestic swine herds in the US and Europe through testing and slaughter programs lead to a general paucity of swine brucellosis research in recent decades. Most literature in the swine brucellosis field was published between 1930 and 1960. However, within the last decade there has been a renewed interest in swine brucellosis which has lead to a small revival in research related to swine brucellosis and *B. suis*. This renewed interest at a time of near eradication of *Brucella* from the US was sparked by an initiative to deal with wildlife reservoirs of *Brucella*. Feral swine are enzootically infected with *B. suis* in the US and are responsible for the remaining outbreaks of brucellosis in domestic swine. Another reason for general renewed interest in *Brucella* related research has been the inclusion of *B. suis*, as well as *B. melitensis* and *B. abortus*, on the US Centers for Disease Control and USDA Select Agents list due to their perceived potential use as biological agents of terrorism and war.9

Virtually all publications dealing with feral swine brucellosis have been mere serological surveys in selected populations. Recent *Brucella* vaccine studies in domestic swine have suggested that the naturally rough mutant cattle vaccine *B. abortus* RB51 (RB51) would be effective in controlling swine brucellosis; however, controlled challenge studies had not been attempted.3,5 The research reported herein was designed to investigate basic characteristics of brucellosis in feral swine and to investigate possible
vaccine candidates for the control of swine brucellosis. Work in this dissertation addressed microbiological and pathological aspects of brucellosis in a feral swine herd. Also, studies in this dissertation assessed the usage of RB51 in domestic and feral swine as well as a newly described naturally rough mutant of *B. suis* as a vaccine in domestic swine.

**Dissertation Organization**

This dissertation is presented in the alternative format and includes a review of the pertinent literature, four manuscripts submitted to three different journals, and general conclusions. Chapter 2 is a review of historical and recent literature dealing with brucellae, swine brucellosis, *B. suis*, *Brucella* vaccines, and feral swine. Chapter 3 is a manuscript submitted to the *Journal of Veterinary Diagnostic Investigation*. This manuscript characterizes a feral swine herd enzootically infected with *Brucella* and attempts to provide information regarding characteristics of isolated *Brucella*, culture and seroprevalence, and lesions of *Brucella* infected animals. Chapter 4 is a manuscript submitted to the journal *Research in Veterinary Science* and reports the results of a vaccine trial with RB51 in an enzootically infected feral swine herd. Chapter 5 is a manuscript published in the *American Journal of Veterinary Research*. This paper reports the results of experiments designed to assess the distribution, clearance, immunogenicity, and efficacy of RB51 in domestic swine. Chapter 6 is a manuscript submitted to the *American Journal of Veterinary Research* which characterizes a newly recognized naturally rough mutant of *B. suis*, *B. suis* 353-1, with respect to its pathogenicity, distribution, clearance, immunogenicity, and efficacy as a vaccine in domestic swine. Chapter 7 includes general conclusions. References cited within each chapter are listed at the end of the respective chapter, and Chapters 3 to 6 are formatted according to the respective journals to which they were submitted.

**References**


CHAPTER 2: LITERATURE REVIEW

Introduction

The brucellae are gram-negative, coccobacillary, aerobic, facultative intracellular alpha-proteobacteria which are known to infect over 80 mammalian species.\(^{107}\) The genus *Brucella* is composed of 8 highly genetically homogeneous species.\(^{117,231,232}\) *B. melitensis* was the first species identified and infects primarily goats and sheep.\(^{35,170}\) *B. abortus* was first identified in cattle\(^ {15}\) but enzootically infects a number of large ruminant species including North American bison (*Bison bison*)\(^ {73}\) and elk (*Cervus elaphus nelsoni*).\(^ {225}\) *B. suis* was first isolated from swine,\(^ {226}\) but other members of the species infect reindeer and caribou (*Rangifer tarandus*) and murine species.\(^ {160}\) *B. ovis* primarily infects sheep and is of lower virulence than the other *Brucella* species.\(^ {37}\) *B. canis* is enzootic in canid species,\(^ {82,134}\) and *B. neotomae* has been identified in the desert wood rat (*Neotoma lepida*) of the American West.\(^ {218}\) The most recently identified *Brucella* species are those which infect marine mammals. Strains which infect seals, sea lions, and related species have been organized into the species *B. pinnipediae*, and those which infect dolphins and related species are now part of the species *B. cetaceae*.\(^ {51,54}\) The genomes of 3 of the species (*B. melitensis*\(^ {76}\), *B. suis*\(^ {195}\), and *B. abortus*\(^ {117}\)) have been completely sequenced, and due to the marked genetic similarity of these three species and sequenced segments of the other five, some have suggested that there should be only one species, *B. melitensis*.\(^ {162,231,232}\) Four members of the genus, *B. melitensis*, *B. suis*, *B. abortus*, and *B. canis* are zoonotic causing disease in humans referred to as undulant fever.\(^ {111}\)

Swine brucellosis occurs at widely variable incidence rates throughout the world in domestic or feral swine populations except in Great Britain and the Scandinavian peninsula where the disease has been eradicated.\(^ {63,146}\) The highest incidence rates of swine brucellosis in domestic swine occur in Central and South America, Asia, particularly China and Southeast Asia, and sub-Saharan Africa.\(^ {86,143,156,200}\) *B. suis* has been essentially eradicated from domestic populations in the US, northern Europe, and Australia; however, *B. suis* is enzootic in feral populations in these locations.\(^ {2,112,151,245}\) Sporadic outbreaks of swine brucellosis in domestic herds in these areas are related to
contact with infected feral swine. Occasional outbreaks of brucellosis in cattle also occur due to *B. suis* infection from feral swine. Pyogranulomatous prostatitis and epididymitis in dogs and cases of fistulous withers in horses have both been caused by *B. suis* infection in areas with enzootic feral swine brucellosis.

*Brucella suis* – biovars and microbiological characteristics

*B. suis*, like the other *Brucella* species, are carboxyphilic, nonmotile, nonhemolytic, partially acid-fast, catalase positive pathogenic bacteria which are easily propagated on nutrient agar, and their propagation is enhanced by enrichment with blood or serum. The strong, almost immediate urease activity of *B. suis* easily allows its differentiation from *B. abortus* and most *B. melitensis* isolates. Lysis by the Tbilisi phage can also be used to differentiate *B. suis* from *B. abortus* and *B. melitensis*. *B. melitensis* is resistant to lysis by the Tbilisi phage at both the recommended test dilution (RTD) and $10^4 \times$ RTD. *B. suis* exhibits intermediate sensitivity to the Tbilisi phage by being resistant to lysis at the RTD but sensitive at $10^4 \times$ RTD. *B. abortus* is the most sensitive of all the brucellae to the Tbilisi phage in that it is lysed at the RTD. *B. ovis* and *B. canis* only exist as rough strains lacking the O-polysaccharide side-chain of the lipopolysaccharide (LPS) molecule. Rough strains of *B. suis* exist and can be differentiated from *B. ovis* due to the obligate carboxyphilic nature of *B. ovis*. *B. suis* can also be easily differentiated from *B. canis* because, like *B. melitensis*, *B. canis* is resistant to lysis by the Tbilisi phage at the RTD. The microbiological characteristics of *B. neotomae* are highly similar to those of *B. suis*; however, these two species can be distinguished by the inability of *B. neotomae* to grow on nutrient agar containing 20 μg/ml thionin.

*B. suis* is subclassified into 5 biovars based on dominant antigens, growth characteristics, and the animal species which they primarily infect. *B. suis* biovar 1, which is found worldwide, is the only biovar to produce hydrogen sulfide, expresses the A antigen, and infects primarily swine. *B. suis* biovar 2 is differentiated from biovar 1 in that it does not produce hydrogen sulfide and is differentiated from biovar 3 in that it does not grow on basic fuchsin containing media. Biovar 2 is primarily found in
Europe, is generally considered to not be infectious to humans, and is maintained in European hares (*Lepis capinensis*) which are considered to be able to transmit the organism from farm to farm.\textsuperscript{24, 49, 222, 223} Biovar 3 primarily infects swine and is also widely distributed throughout the world. Biovar 3 is the only biovar to consistently grow on agar containing basic fuchsin and expresses the A antigen dominantly.\textsuperscript{7, 199} Biovar 4 is maintained in reindeer and caribou herds in the subarctic regions of the Northern Hemisphere, and natural infection of biovar 4 has never been recognized in swine. Biovar 4’s distinguishing bacteriological characteristic is that it coexpresses the A and M antigens.\textsuperscript{7, 199} Biovar 5 is a minor biovar of *B. suis* which has been isolated from several rodent species and is distinguished from the other biovars by dominantly expressing the M antigen.\textsuperscript{7, 60, 62, 199}

**Brucella suis infection: clinical disease and pathogenesis**

*B. suis* is largely still thought of as a primarily abortifacient organism. Abortion is indeed associated with epizootics of swine brucellosis; however, abortion is a minor component of the disease presentation of *B. suis* infection. As early as 1931, it was recognized by Johnson and Huddleson\textsuperscript{132} that swine brucellosis was not primarily an “abortion disease”. Johnson and Huddleson blame this misconception on the fact that *B. suis* was first isolated from an aborted pig fetus,\textsuperscript{226} “Since *Brucella* was isolated by Traum from fetuses expelled prematurely from sows, the belief has been widely prevalent that *Brucella* disease in swine is essentially an abortion disease. Our studies of the natural course of the disease in several large groups of hogs in the state of Michigan have failed to confirm this belief.”\textsuperscript{132} Hutchings\textsuperscript{128} also realized that a great emphasis was being placed on reproducing abortions in the experimental evaluation of *B. suis* infections. He suggested that evaluating resistance to infection rather than preventing abortions which were extremely difficult to produce with experimental infection, was more important in evaluating control measures of *B. suis* transmission.

*B. suis* can infect swine by a variety of exposure routes. Unlike the other species of *Brucella*, *B. suis* is venereally transmitted.\textsuperscript{146, 149} Ingestion of infected material is the other major route of natural exposure; however, inhalatory exposure is also capable of
causing infection. Several studies have addressed the minimal infectious dose of *B. suis*. Models using *B. suis* biovar 3 (strain 644) found that dosages of $10^5$, $10^7$, and $10^9$ colony forming units (CFU) were all capable of infecting 100% of animals challenged while reduced infection rates were achieved with a dose of $10^3$ CFU. Experimental studies managed to transmit *B. suis* to swine, as well as *B. abortus* to cattle, via insect vectors (*Stomoxys calcitrans*); however, there is little evidence that vector transmission occurs with natural infection.

After initial exposure to *B. suis*, there is uptake of bacteria and delivery to draining lymph nodes, both via an incompletely understood process. Bacteremia then occurs, and the persistence of the bacteremia is quite variable and has been demonstrated to last 1 week to 34 months in infected sows. Systemic infection of lymphoid organs closely follows bacteremia. Initial infection is not associated with fever except in some boars which eventually succumb to severe pyogranulomatous lesions. Changes in leukograms also do not occur with acute and chronic *B. suis* infection.

Reproductive losses in sows have been recognized to occur as early as 22 days after successful insemination with *B. suis* contaminated semen, and irregular return to estrus presents in those cases 30 to 45 days after insemination. When abortions occur in mid to late gestation, they are associated with the *B. suis* infection being acquired after day 40 of pregnancy. Uterine infection and vaginal shedding of *B. suis* is highly variable and has been reported to occur from one to 30 months after initial infection. Infertility in infected sows has been shown to be related to the duration of infection and the extent of lesions in the uterus.

*B. suis* is often found in the semen from infected boars. Boars shedding *B. suis* in their semen frequently are associated with lower conception rates and lower live pigs born per litter than noninfected boars. This decrease in reproductive efficiency is not necessarily associated with decreases in semen quality. Infection in reproductive organs of males has been shown to persist for at least three to four years in some individuals. The extent of infertility in boars is related to the distribution of infection and lesions. Individuals with unilateral involvement of the accessory sexual organs rarely exhibit decreases in fertility or libido yet disseminate large numbers of organisms
in their semen. Individuals with testicular involvement or bilateral involvement of the accessory sexual organs exhibit marked decreases in fertility and possibly libido.\textsuperscript{126}

Clinical signs associated with \textit{B. suis} infection in prepubescent swine are rare and include swollen joints and lameness which are associated with bacteremia. Pigs born to infected sows are often infected with \textit{B. suis} and exhibit markedly high neonatal mortality rates.\textsuperscript{68,128,129} Most pigs which survive have been shown to become blood culture negative by 3 months of age and achieve systemic clearance by six months of age.\textsuperscript{109,127} In larger studies, however, low numbers of animals were found to become chronically infected with \textit{B. suis} when exposed as nursing pigs.\textsuperscript{147} Manthei found that about 8\% of 230 pigs exposed to \textit{B. suis} while nursing were blood culture positive beyond 3 months of age, and about 2.5\% were tissue positive at slaughter after two years of age.\textsuperscript{150} Systemic clearance appears to be more protracted in sexually mature swine. In sexually mature females, systemic clearance appears to occur between two and six months after infection as the \textit{Brucella} recovery rate from necropsy tissue declined from 94.4\% to 23.5\% in this period.\textsuperscript{80} However, there was no change in recovery rate thereafter with recovery rates remaining around 25\% through 42 months after infection.\textsuperscript{80} Sexually mature males become chronically infected at a significantly higher rate than females. Six months after initial experimental infection 66.7\% of sexually mature males remained infected as determined by \textit{Brucella} recovery from necropsy samples.\textsuperscript{80} This rate only declined to 50\% at 42 months after initial infection.\textsuperscript{80}

Natural resistance to \textit{B. suis} infection has been reported in certain lines of swine.\textsuperscript{39,40} Some have suggested that this is related to genetic differences related to the natural resistance associated macrophage protein (NRAMP1).\textsuperscript{228} The \textit{Nramp1} gene has been cloned in pigs, and it has been shown that NRAMP1 is strongly expressed in macrophages and neutrophils following nonspecific stimulation with LPS.\textsuperscript{244}

The cellular events of \textit{Brucella} infections have been explored in both phagocytic and nonphagocytic cells.\textsuperscript{110} The events involved with mucosal penetration are poorly understood, but after penetration \textit{Brucella} cells are taken up by M cells, macrophages, and neutrophils via a zipper-like phagocytosis.\textsuperscript{1} This phagocytosis is mediated by complement or Fce receptors, and lectin and fibronectin receptors mediate uptake in
nonprofessional phagocytic cells. Internalization of Brucella also appears to be dependent on the brucellar two component system BvrS-BvR which encodes for a histidine kinase sensor and its regulator protein. This system seems to regulate the expression of several outer membrane proteins. Once internalized, intracellular survival of Brucella is dependent on avoiding lysosome fusion with the phagosome. After internalization the phagosome membrane contains both lysosomal-associated membrane protein 1 (LAMP1) as well as sec61β and calreticulin, both endoplasmic reticulum markers. The phagosome is, however, deficient of lysosomal hydrolase cathepsin D a luminal marker of lysosomes. Early acidification of the phagosome, although detrimental to the short term replication of Brucella, has been shown to be necessary for the ultimate survival of Brucella within macrophages. The final replicative compartment of Brucella is considered to be the endoplasmic reticulum as LAMP1 is lost but sec61β labeling remains.

Immunity

Most of what is known about the immune response to natural Brucella infection and responses to Brucella vaccines has been discovered through studies in mice, cultured, cells, and isolated peripheral cells from humans with brucellosis. Early infection of macrophages with B. suis induces an innate response of cytokine production. Interleukin-1 (IL-1), IL-6, and IL-10 as well as the neutrophil-stimulating chemokine IL-8 are produced early in the infection of the macrophage. Apoptosis is downregulated in human macrophages which are infected with B. suis. Intracellular survival and replication of the B. suis appears to be necessary for this downregulation. An upregulation of the antiapoptotic gene bcl-2 has been shown to be associated with this occurrence; however, a soluble factor appears to be involved in the signaling process as supernatant from infected cells can downregulate apoptosis in noninfected cells. B. suis infection of macrophages also decreases their responsiveness to Fas ligand (FasL) and interferon gamma (IFN-γ) induced apoptosis. Within B. suis infected macrophages there is also a downregulation of tumor necrosis factor-alpha (TNFα) production which has been reported to be dependent on the 25 kDa outer membrane protein (Omp25).
Downregulation of TNF-α appears to make the cells less responsive to IL-12 and IFN-γ. 84, 166 Humans infected with *B. suis* have a dramatic increase in circulating γ/δ T cells which are activated by soluble factors from *B. suis*. 27 After activation, the T cells produce TNF-α and IFN-γ. 189, 190 Natural killer (NK) cells from humans have also been shown to decrease intramacrophagic replication of *B. suis* via a contact dependent mechanism which is enhanced by IL-2. 85 In addition to the contact-dependent mechanism, NK cells appear to be activated as they produce IFN-γ and TNF-α which also likely enhances their activity and the activity of the macrophages in clearing *B. suis*. 8

Murine models of *Brucella* infection have shown that a type 1 T helper (Th1) cell cytokine response is necessary for clearance of infection. 13, 97 This Th1 response has been shown to be elicited by triggering toll-like receptor 9 (TLR9). 125 The ligand for TLR9 is unmethylated DNA, and heat-killed *B. abortus* has been shown to simulate TLR9 and induce a Th1 cascade. TLR2 and TLR4 which are stimulated by LPS have been shown to not be necessary for clearance of *B. abortus*. 236 The Th1 response to *Brucella* infection in mice is characterized by a strong IFN-γ response in splenocytes. 98 Blockage of the Th1 cytokine cascade did not shift the response of mice to a Th2 response as IL-4 production was not elevated in the mouse splenocyte. 97 Studies in BALB/c mice, which have a marked lag in the upregulation of the Th1 response after *Brucella* infection, show that CD8+ T cells and TNF-α contribute to the control of *Brucella* infection but are not satisfactory in themselves in inducing full clearance of the organism. 97, 174 However, despite a lack of measurable IL-4, blockage of both IL-4 and IL-10 pathways resulted in decreases in *Brucella* infection. 13, 96

Like other species of *Brucella* in their respective animal hosts, there is little evidence that humoral immunity is protective in preventing infection or reinfection with *B. suis*. 14, 130, 164, 239 Sows with low serum agglutination titers by the standard tube test (STT) were easily infected with *B. suis* when given a dose of 10^7 CFU by the conjunctival route. 44

Cell-mediated immune responses of swine to *B. suis* infection have not been thoroughly investigated. Cultured peripheral blood mononuclear cells (PBMCs) from swine naturally infected with *B. suis* were shown to proliferate with stimulation from
soluble *B. suis* antigens. PBMC proliferative responses were not correlated to serological titers on an individual animal basis; however, the two had a high degree of correlation on a group basis. Although there is little information on immune responses related to *Brucella* infection in swine, IFN-γ has been shown to be the most central factor in Th1 immune responses related to clearance of intracellular pathogens in swine.

**Lesions associated with *Brucella suis* infection**

Lesions in lymph nodes are frequently reported in swine infected with *B. suis*. Variable degrees of lymphadenopathy are usually the only gross lesion evident in lymph nodes; however, occasionally purulent exudate or organized abscesses are recognized. Microscopically, lymph nodes have variable numbers of inflammatory cells consisting of neutrophils, epithelioid macrophages, and multinucleated giant cells and foci of coagulative, liquefactive, or caseous necrosis. Lymphoid hyperplasia has also been reported in culture positive lymph nodes. For all lymph node lesions, the most commonly reported affected lymph nodes are gastrohepatic, medial retropharyngeal, parotid, mandibular, tracheobronchial, and splenic. Pyogranulomatous and necrotizing lesions similar to those reported in lymph nodes have also been reported in spleens of *B. suis* infected swine. Reported hepatic lesions range from microscopic random or portal accumulations of lymphocytes, plasma cells, macrophages, and neutrophils to grossly visible foci of caseous necrosis which are microscopically characterized as multifocal granulomas or pyogranulomas with central necrosis. Fibrosing perihepatitis has also been reported to be associated with *B. suis* infection.

Lesions in male reproductive organs of swine are well documented with *B. suis* infection. Gross lesions are most commonly reported in seminal vesicles. Affected seminal vesicles are markedly enlarged due to an increase in fibrous connective tissue separating individual lobules which may contain purulent exudate. More acute lesions may be mere abscesses without fibrosis. Histologically affected seminal vesicles contain abundant fibrous tissue and numerous lymphocytes, plasma cells, macrophages, and multinucleated giant cells with some lesions being neutrophil rich. The epithelium of ducts within affected seminal vesicles is often necrotic or totally denuded,
and there are occasional areas of caseous or liquefactive necrosis throughout the parenchyma. Similar lesions may also be found in the prostate, bulbourethral gland, epididymis, and testis. With chronicity, lesions in the male secondary reproductive organs progress to a markedly atrophic and fibrotic appearance. Testicular inflammation ranges from mild infiltrates of lymphocytes, plasma cells, macrophages, and neutrophils to well organized pyogranulomas. Large areas of necrosis have also been noted in infected testes. Seminiferous tubules in affected testes may be normal or exhibit marked atrophy with limited spermatozoa formation.

Lesions in female reproductive organs are recognized most frequently with *B. suis* biovar 2. The classical gross lesion associated with *B. suis* biovar 2 has been termed miliary metritis due to its marked multifocal, widely disseminated distribution and has been reported to be present in as much as 47% of sows and gilts during an epizootic. The grossly visible nodules are composed of caseous necrosis surrounded by abundant numbers of neutrophils alone or neutrophils with epithelioid macrophages and multinucleated giant cells. Similar lesions are described in the kidneys and spleens of *B. suis* biovar 2 infected swine. The swine lesions are highly similar to the lesions observed in the European hare which is considered a natural host reservoir and responsible for European outbreaks of swine brucellosis. Affected hares exhibit nodules of caseous necrosis surrounded by pyogranulomatous inflammation in the spleen, lung, liver, sacculus rotundus, uterus, testis, epididymis, and mammary gland. Natural and experimental infection with biovars 1 and 3 in sows and gilts occasionally produce grossly visible purulent to fibrinopurulent endometritis although frank vaginal purulent discharge is rarely recognized. Cystic endometritis and catarrhal endometritis have also been reported to be features of *B. suis* biovar 1 infection. Microscopically uterine lesions with *B. suis* biovars 1 and 3 range from multifocal endometrial aggregates of lymphocytes, plasma cells, neutrophils, and macrophages with or without necrosis to diffuse purulent endometritis with necrosis.

Lesions in placentas and tissues from aborted fetuses have been reported to be rare. Lesions which have been reported include grossly visible hyperemia, edema, and/or hemorrhages within placenta, increased subcutaneous and peritoneal fluids which
may be hemorrhagic, and microscopically observable purulent placentitis with or without necrosis.  

Bone lesions have been reported commonly in vertebrae and less commonly in long bones. Vertebral lesions are often grossly visible and consist of pyogranulomatous osteomyelitis with marked necrosis and with or without intervertebral disk involvement. When diskospondylitis is present, there is often accompanying impingement of the spinal cord with marked atrophy of particularly white matter tracts. Diskospondylitis development from *B. suis* infection in swine appears to have a predilection for the lumbar and lumbosacral areas. Bone lesions also have been reported to have notable neovascularization, bone sequestra are common. Less frequent lesions associated with *B. suis* infection in swine include subcutaneous abscesses, lymphohistiocytic inflammatory aggregates in the lung, pyogranulomatous meningoencephalitis, and lymphocytic and purulent adrenalitis.

## Diagnosis of swine brucellosis

### Serological assays

All standard serological assays used in the diagnosis of *B. suis* infection utilize O-polysaccharide side-chain of the lipopolysaccharide (LPS) molecule from *B. abortus*; thus, none are designed specifically for *B. suis*. However, limited early studies with serological assays utilizing *B. suis* specific antigens reported no appreciable differences over assays which utilized the standard *B. abortus* antigens. The O side-chain also contains the A and M antigens which are utilized for *Brucella* speciation and biotyping. The O side-chain is a homopolymer of approximately 100 residues of 4-formamido-4, 6-dideoxymannose linked solely in an \( \alpha-1, 2 \) conformation in the A dominant strains and in an \( \alpha-1, 2 \) conformation with every fifth residue linked in an \( \alpha-1, 3 \) conformation in the M dominant strains. The 4-amino-4, 6 dideoxymannose epitope is responsible for the *Brucella* antigenic crossreactivity with LPS from *Yersinia enterocolitica* O:9, *Escherichia coli* O:157; *Escherichia hermannii*, *Salmonella* O:30 serogroup; *Vibrio cholerae* O:1, and *Stenotrophomonas maltophilia*. Rough mutants of *Brucella* express
LPS in which the O side-chain is totally absent or greatly reduced; therefore infection with these mutants results in seronegative animals.\(^{180}\)

The literature has great discrepancies in the utility of serology in the diagnosis of \textit{B. suis} infection. Certainly as a herd diagnostic tool, standard serological techniques are adequate; however, their ability to identify individual, infected animals appears to be poor. Most studies dealing with determining sensitivity of individual serological assays utilize sera from acutely infected individuals. Sensitivity rates reported for individual assays are as follows: STT: 51.1\%-100\%;\(^{103, 206}\) mercaptoethanol test (2-ME): 38.5\%-100\%);\(^{103, 143, 194}\) rivanol test: 23.1\%-100\%);\(^{99, 103, 143, 152}\) complement fixation test: 49.1\%-100\%\(^{99, 103, 143, 206, 173}\) card test: 20\%-100.0\%);\(^{103, 143, 206}\) and buffered plate antigen (BPA) test: 61.0\%-77.1\%.\(^{99, 152, 173}\) Specificity rates are as follows: STT: 62.0\%-100\%\(^{99, 143, 206}\) 2-ME: 81.1\%-100\%;\(^{143, 194}\) rivanol test 74.0\%-100\%;\(^{99, 143, 152}\) complement fixation test: 86.0\%-100\%;\(^{99, 143, 152, 206}\) card test 76.0\%-92.0\%;\(^{99, 152, 206}\) and BPA test: 90.0\%-95.9\%.\(^{99, 173}\) The specificity of all standard serological assays is considered to be low with the presence of confounding infections particularly infections with \textit{Yersinia enterocolitica} serogroup O:9.\(^{241}\) The fluorescent polarization assay (FPA) is the most recently developed serological tool for \textit{Brucella} serological diagnosis.\(^{172}\) The reported sensitivity of the FPA assay is 63.0\%-98.9\%, and the specificity is reported to be 55.0\%-99.9\%.\(^{152, 173, 194}\) The FPA studies utilized sera which was previously determined to be positive by the BPAT or 2-ME assays; therefore, the sensitivity values are likely inappropriately high.

**Delayed-type hypersensitivity**

Intradermal skin testing has been used to diagnose swine brucellosis and has been reported to be as effective on a herd basis as serological evaluation.\(^{213}\) Original brucellins were prepared from smooth strains of \textit{B. melitensis} and \textit{B. abortus}.\(^{191}\) Manthei examined three brucellins prepared from \textit{B. suis} in swine: a phosphatide fraction, a purified culture filtrate, and a soluble nucleoprotein fraction.\(^{149}\) The sensitivity of the delayed-type hypersensitivity skin test using these three fractions was reported to be slightly higher than standard serological assays; however, like the serological assays, the skin test was not successful in identifying all individually infected animals.\(^{149}\) Newer
brucellins have been prepared from rough strains of *B. melitensis* with reports of higher specificities compared to the smooth brucellins.\textsuperscript{133}

**Bacteriological culture**

Bacteriological culture is, of course, the definitive method for diagnosis of swine brucellosis. Deyoe and Manthei cultured necropsy tissue samples from 147 swine experimentally infected with *B. suis* biovar 1 and euthanized one to 52 months after infection.\textsuperscript{81} Lymph nodes were the most consistently culture positive organs (75.2%), and the most consistently positive lymph nodes were mandibular, gastrohepatic, internal iliac, medial retropharyngeal, and parotid; however, all lymph nodes examined yielded *Brucella* growth at some incidence rate.\textsuperscript{81} Bacteriological culture of only the mandibular, medial retropharyngeal, gastrohepatic, and internal iliac lymph nodes would have identified 91% of the total culture positive animals.\textsuperscript{81} Male reproductive organs (37.2%) were more consistently positive than uterus (28.9%). Other culture positive tissues and fluids included: spleen (38.7%), lung (36.8%), liver (31.8%), blood (30.1%), bone marrow (25.0%), joint fluid (23.9%), urine (20.8%) tonsil (14.8%), kidney (14.5%), salivary gland (12.2%), adrenal (11.7%), brain (7.5%), and ileocecal valve (3.7%).\textsuperscript{81}

**Molecular techniques**

Polymerase chain reaction (PCR) techniques have been employed for culture identification and *Brucella* species differentiation.\textsuperscript{31} These techniques have been used mostly to identify *Brucella* which has first been cultured. While several studies have incorporated PCR to identify *Brucella* directly from tissue, there has been limited investigation into the sensitivity of PCR identification of *Brucella* tissues, blood, or fluids from infected animals.\textsuperscript{8, 69, 114, 220} In the few studies which have looked at sensitivity of PCR assays, sensitivity was often low compared to bacteriologic culture.\textsuperscript{243}

PCR assays have been developed that are *Brucella* genus specific. These assays have been based on the *omp2A*,\textsuperscript{100} *omp43*,\textsuperscript{94} 16S rRNA,\textsuperscript{119, 207} 16S-23S intergenic region,\textsuperscript{202} and *BCSP31* genes.\textsuperscript{12} PCR assays which allow species and biovar differentiation have been developed based on the insertion sequence (IS) 711.\textsuperscript{33, 118} The IS711 has allowed differentiation of *B. abortus* biovars 1, 2, and 4; *B. abortus* strain RB51; *B. melitensis*, *B. ovis*, and *B. suis* biovar 1.\textsuperscript{32, 33, 92} The *omp2A* gene can be
targeted by PCR to distinguish \( B.\) \textit{abortus} from other \textit{Brucella} species.\textsuperscript{142} \textit{Omp2A} PCR combined with restriction fragment length polymorphism (RFLP) analysis can discriminate most species of \textit{Brucella}.\textsuperscript{54} Additional PCR-RFLP assays which can discriminate \textit{Brucella} species have targeted the \textit{omp25},\textsuperscript{52} \textit{dnaK},\textsuperscript{53} and \textit{omp31} genes.\textsuperscript{233} The vaccine strain \( B.\) \textit{abortus} S19 contains a stable deletion in the \textit{eri} gene which allows its differentiation by PCR from other \textit{Brucella} possible.\textsuperscript{209}

**Immunoochemical techniques**

Immunoochemical techniques have been applied to the diagnosis of \( B.\) \textit{suis} infection in swine; however, these techniques have been found to be far inferior to bacteriological culture.\textsuperscript{161} Meador reported an immunohistochemical assay for the diagnosis of \( B.\) \textit{abortus} in cattle, goats, and mice; however, it has never been applied to \textit{Brucella} infected swine.\textsuperscript{159} The sensitivity of a similar immunohistochemical technique was determined to be 82\% when applied only to lochial smears.\textsuperscript{3} Deyoe reported a sensitivity rate of 14.3\% for immunofluorescence techniques utilizing polyclonal antibodies applied to a variety of frozen tissue sections.\textsuperscript{80}

**Experimental infection of swine with other \textit{Brucella} species**

Limited studies of experimental infection of swine with \( B.\) \textit{suis} biovar 4 have shown that swine develop an infection of draining lymph nodes which does not progress to a similar distribution and disease as \( B.\) \textit{suis} biovars 1, 2, or 3.\textsuperscript{18, 79, 80, 149} The results of various studies of experimental infection of swine with \( B.\) \textit{abortus} are contradictory exhibiting widely variable results. Most studies show that the majority of swine do not succumb to systemic infection with \( B.\) \textit{abortus}.\textsuperscript{18, 188} Although natural infections of swine with \( B.\) \textit{abortus} have been reported,\textsuperscript{70, 154, 155} when experimentally infected, \( B.\) \textit{abortus} can usually only be recovered from the draining lymph node from a variable number of animals for a short duration of time.\textsuperscript{146} Additional experimental infection studies with \( B.\) \textit{abortus} in swine showed that most swine do not seroconvert to smooth strains of \( B.\) \textit{abortus} when orally challenged.\textsuperscript{18} A low percentage of swine challenged with a smooth strain of \( B.\) \textit{abortus} seroconverted when infected by subcutaneous, intramammary, or intravenous routes.\textsuperscript{18, 163} Experimentally exposing swine to naturally infected cattle or
feeding swine with *B. abortus* contaminated milk produced a very low rate of seroconversion and infection with only recovery of *B. abortus* from draining lymph nodes.\textsuperscript{108}

There are several early reports of natural *B. melitensis* in swine;\textsuperscript{72, 155} however, most, if not all of these isolates were actually *B. suis* biovar 3. The characteristics of the swine *B. melitensis* isolates described in the early literature are exactly those of *B. suis* biovar 3 and really do not fit those of classical *B. melitensis* organisms as all the swine isolates are fast urease positive.\textsuperscript{20} Therefore, the early artificial infection study of *B. melitensis* in swine is suspect; in that study, swine were infected with swine isolates of *B. melitensis* which were undoubtedly *B. suis* biovar 3.\textsuperscript{121} Subsequent experimental infections of swine with *B. melitensis* by oral and subcutaneous routes produced similar results as experimental infections with *B. abortus*; *B. melitensis* was only isolated from 20% or less of infected animals and only from a single lymph node, presumably the draining node, from each culture positive animal.\textsuperscript{20, 188} Some gilts experimentally infected with high doses of *B. melitensis* did develop bacteremia which lasted seven to 13 weeks after initial infection.\textsuperscript{20}

Experimental, intravenous infection of swine with *B. neotomae* produced seroconversion; however, experimentally infected animals failed to develop infection of lymphoid organs which lasted the nine weeks of the study.\textsuperscript{19}

**Brucella vaccines used to control brucellosis**

*Brucella abortus* strain 19

*B. abortus* strain 19 (S19) has been the most widely used vaccine for the prevention and control of cattle brucellosis.\textsuperscript{212} S19 was first isolated from the milk of a Jersey cow in 1923, and its reduced virulence was apparently attained from the isolate being kept at room temperature for over one year.\textsuperscript{36} S19 is a smooth, biovar 1 strain which is sensitive to penicillin and cannot grow in the presence of 1.0 mg/ml erythritol, both properties which allow it to be differentiated from field strains of *B. abortus*; however, there is a high reversion rate for growth on erythritol.\textsuperscript{7} Being a smooth strain, vaccination with S19 induces a humoral response which causes vaccinated animals to be
seroreactors on standard Brucella serological assays. However, when S19 is employed as only a calfhood vaccine, serum antibodies decline by the time sexual maturity is reached allowing the vaccine to be an integral part of brucellosis control without preventing the detection of animals infected with field strains of B. abortus in the cow herd. Although S19 is of reduced virulence, it can induce abortion at a variable rate (1-14%) when pregnant animals are vaccinated. S19 may also fail to be cleared in a very low percentage of vaccinated animals. These S19-chronically infected animals may succumb to abortion in adulthood. S19 can be cultured from placenta and fetal tissues from abortions from S19-chronically infected cows. Type III hypersensitivities expressed as multicentric arthropathy/arthritis has also been reported as a side-effect of S19 vaccination.

Numerous trials have examined the efficacy the S19 vaccine in cattle. Experimental efforts were made to determine the minimal effective dosage and minimal age of vaccination. These trials all evaluated vaccine efficacy based on the pregnant female challenge model which evaluates efficacy on the basis of abortion and Brucella recovery from maternal and fetal/neonatal tissues; although there is great discrepancy in the results of these studies, they suggest that abortion may be prevented when calves are vaccinated as young as four months of age but not one month of age. Protection based on fetal infection is much more variable, and the optimal vaccine dose and age of vaccination is much more difficult to determine in light of the variable challenge dosages. S19 has been used in elk (Cervus elaphus nelsoni) as a ballistically administered parenteral vaccination in Wyoming since the 1980s. The biobullet vaccine system used in elk delivered dosages of 5.6-7.6 × 10^9 CFU. Several experimental trials have shown a decrease of approximately 30% in the abortion rate in vaccinates compared to nonvaccinated controls.

Brucella abortus strain 45/20

The parent strain of B. abortus 45/20, 45/0, was originally isolated from a cow in Great Britain in 1922. B. abortus strain 45/20 is a rough strain which was derived from 20 passages of strain 45/0 in guinea pigs. However, recent monoclonal antibody studies suggest that the rough variants of 45/20 produce O-side chain.
a rough strain, 45/20 was attenuated; however, when used as a vaccine 45/20 was found to revert to smooth phenotype which had increased virulence.\textsuperscript{90,221} This led to the decreased usage of live 45/20 as a vaccine; however, killed, adjuvanted 45/20 became a preferred vaccine in certain cattle brucellosis control programs.\textsuperscript{205} Despite being derived from rough 45/20, approximately 1\% of cattle vaccinated with the killed, adjuvanted 45/20 vaccine become serological reactors to standard \textit{Brucella} serological assays.\textsuperscript{205} The induction of \textit{Brucella} agglutinating antibodies was more marked in animals which had been previously vaccinated with S19,\textsuperscript{205} and the overall incidence rate of developing \textit{Brucella} agglutinating antibodies after 45/20 vaccination was reported to be highly variable among lots of vaccine.\textsuperscript{212} Vaccine protocols which employed killed, adjuvanted 45/20 usually used 2 doses of the vaccine at an interval of six weeks to 3 months with yearly booster vaccination considered necessary by some.\textsuperscript{116,169} However, studies with single dose protocols found similar protection to the two dose protocol.\textsuperscript{6} The induction of cell-mediated immune responses by killed, adjuvanted 45/20 vaccine has been reported to be superior to that of S19,\textsuperscript{50} and protective responses have been reported to be at least equal those of S19.\textsuperscript{116,205} Killed, adjuvanted 45/20 vaccine has also been used in protocols with S19; S19 was used as a calfhood vaccine and 45/20 was used to boost adult cattle.\textsuperscript{169}

Killed \textit{Brucella} vaccines have been investigated in reindeer for the prevention of \textit{B. suis} biovar 4 transmission.\textsuperscript{83} Killed, adjuvanted preparations of \textit{B. abortus} strain 45/20 were found to be efficacious when vaccinated reindeer were challenged 90 days after vaccination. Efficacy was assessed on the basis of a delay in seroconversion, decreased abortion rate, and decreased \textit{Brucella} culture from necropsy tissues from vaccinated females and their offspring.\textsuperscript{83}

\textbf{\textit{Brucella abortus} strain RB51}

\textit{B. abortus} strain RB51 (RB51) is a naturally rough mutant of the challenge strain \textit{B. abortus} 2308. RB51 is rifampimycin resistant, a trait which allows its selective isolation from mixed or contaminated culture samples.\textsuperscript{123} Being a rough strain, RB51 does not induce the production of antibodies which react in standard \textit{Brucella} serological assays\textsuperscript{180,216} despite recent evidence showing that RB51 synthesizes a low level of M-like
However, there is evidence that animals may become serological reactor to standard Brucella serological assays after an anamnestic response from booster vaccination. RB51 is of low virulence as determined by experimental infection of cattle, mice, and guinea pigs and does not induce abortion when pregnant cattle and bison are vaccinated. RB51 can also be distinguished from other strains of B. abortus by molecular methods due to positions of IS711 elements.

The standard vaccine dosage of RB51 used in the US is $2.0 \times 10^{10}$ CFU RB51. This dosage is associated with clearance in most animals by week 14 after vaccination, significant humoral responses, and strong cell-mediated immune responses in both cattle and bison (Bison bison). These responses have been associated with significant protection from challenge with virulent B. abortus. Studies dealing with RB51 in nontarget species indicate that it is relatively safe for black bears (Ursus americanus), Richardson’s ground squirrels (Spermophilus richardsonii), deer mice (Peromyscus maniculatus), prairie voles (Microtus ochrogaster), ravens (Corvus corax), bighorn sheep (Ovis Canadensis), pronghorn antelope (Antilocapra americana), mule deer (Odocoileus heminonus), moose (Alces alces shirasa), and coyotes (Canis latrans); thus, there is great interest in utilizing this vaccine as a wildlife vaccine, particularly in bison. The induction of protective immunity by oral administration of RB51 in mice and cattle has also spiked interest in utilizing RB51 as an oral wildlife vaccine.

When elk are vaccinated with RB51, they respond with a very intense antibody production but appear to have an increased clearance time and increased time of bacteremia compared to other species. Cell mediated immune responses to RB51 and to lymphocyte mitogens were largely due to proliferation of B lymphocytes in elk, and vaccinated elk showed a lack of protection when challenged with virulent B. abortus.

**Brucella melitensis strain Rev 1**

*B. melitensis* strain Rev 1 (Rev 1) is a smooth, attenuated strain which is resistant to streptomycin and has been used as a vaccine in goats and sheep. Rev 1 is avirulent in rams; however, it will induce abortion in does and ewes if the vaccine is administered.
during pregnancy.\textsuperscript{4,16,28} Like S19, vaccination with Rev 1 produces serological reactors which may interfere with surveillance if vaccination is not limited to a defined age group. Although never employed to control cattle brucellosis as part of a large scale control program, studies indicate that Rev 1 offers better protection than S19 in cattle.\textsuperscript{101,124}

**Brucella vaccine trials in swine**

Several vaccine trials have been performed with *B. abortus* strain 19 (S19) in swine.\textsuperscript{122,146} Manthei evaluated subcutaneous dosages of $1.0 \times 10^{11}$ and $2.0 \times 10^{11}$ CFU in gilts.\textsuperscript{146} The vaccinated gilts were challenged with virulent *B. suis* six months after initial vaccination. There was no protection noted on the basis of bacterial recovery from sow tissues, and vaccinated sows actually had a 21.2% increase in *Brucella* recovery compared to nonvaccinated controls.\textsuperscript{146} Hoerlein conducted several studies using S19 which was commercially available; his results were consistent with Manthei in that there was no apparent protection induced by S19 in preventing infection with virulent *B. suis*.\textsuperscript{122} Additional studies evaluated the S19 vaccine in the control of swine brucellosis under natural field exposure.\textsuperscript{136} S19 failed to show improvement of vaccinated animals on the basis of serological conversion, bacteriological culture of *B. suis*, and reproductive efficiency.\textsuperscript{136} Swine vaccinated with S19 were shown to seroconvert to *B. abortus* antigens with both IgM and IgG production.\textsuperscript{201}

Several studies were performed with a compound vaccine consisting of live *B. abortus* strain Old Bang and LPS antigen from *B. suis*.\textsuperscript{45,46,47} The vaccine was reported to be nonpathogenic in swine and transmission of the vaccine strain among swine was not detected.\textsuperscript{47} Vaccine trials with the *B. abortus* Old Bang/*B. suis* LPS vaccine concluded that it was significantly efficacious based on reduction in *Brucella* recovery and abortion.\textsuperscript{45,46,47} More controlled experiments with defined challenge doses of virulent *B. suis* in guinea pigs failed to show any efficacy of the *B. abortus* Old Bang/*B. suis* LPS vaccine compared to nonvaccinated controls.\textsuperscript{102}

Manthei also evaluated the King 8 strain of *B. suis* biovar 1 which originated in Australia and was reported to be a reduced virulence strain.\textsuperscript{146} Gilts received subcutaneous dosages of $1.0 \times 10^{11}$ CFU and $2.0 \times 10^{11}$ CFU. When challenged at six
months after initial vaccination, vaccinated gilts had a 45.8% reduction in *Brucella* recovery.\textsuperscript{146} Manthei concluded that immunity was too short-lived for the vaccine to be useful because in a second similar experiment where gilts were challenged at 24 months after initial vaccination there was no difference in *Brucella* recovery between vaccinated and control groups.\textsuperscript{146} However, the challenge in the long term King 8 experiment appeared to be stronger as *Brucella* was recovered from 100% of the controls as compared to 45.4%-80.0% in Manthei’s previous swine vaccine experiments.\textsuperscript{146} Others claimed that further experiments with the King 8 strain were not pursued due to concerns that it would revert to full virulence and concerns about its likely pathogenicity to humans.\textsuperscript{71, 79}

Edens and Foster examined LPS containing extracts of *B. suis* as vaccine preparations in swine.\textsuperscript{88} Vaccines were prepared from LPS extracts which were treated or not treated with lysozyme.\textsuperscript{88} An additional vaccine was prepared by conjugating the lysozyme treated LPS extract to bovine gamma globulin.\textsuperscript{88} Vaccinated swine were challenged with virulent *B. suis* 42 days after initial vaccination.\textsuperscript{88} All vaccine preparations were found to protect the swine from virulent challenge based on *Brucella* recovery from necropsy tissues.\textsuperscript{88} The group receiving the gamma globulin conjugated preparation of lysozyme treated LPS showed the most drastic reduction in *Brucella* recovery.\textsuperscript{88} Despite initial favorable results using *B. suis* LPS extracts as a vaccine, it appears that further investigations were not pursued.

*B. suis* strain 2 is a vaccine strain which has been reported to have been used in swine, sheep, goats, and cattle in China since 1971,\textsuperscript{242} however, there is very little knowledge or experience with this vaccine outside of that country. Strain 2 was reported to be attenuated by serial passage on culture media for a year attaining a virulence similar to S19, but it is a smooth strain of *B. suis* biovar 1.\textsuperscript{242} Strain 2 was reported to be nonpathogenic in swine and was reported to be cleared in both males and females by 45 days after vaccination; however, Strain 2 induced abortions in sheep and goats with parenteral administration but not with oral administration.\textsuperscript{242} The attenuation of Strain 2 for swine and guinea pigs was also reported to be very stable with no reversion of virulence when serially passaged in swine, guinea pigs, sheep, and goats.\textsuperscript{242} Subsequent
studies in mice found that Strain 2 was of lower virulence than either S19 or Rev 1 as measured by vaccine strain recovery. Strain 2 has been used in brucellosis control programs for swine, ovine, and caprine brucellosis in China. In swine it has been investigated as part of a multifactorial control program. Vaccination with Strain 2 was accompanied by culling of all females which aborted. The serological prevalence rates on the test farms were reported to drop from 62.8% to 2.4% on one farm and from 75% to 0% on another farm. Others who have paraphrased Chinese studies of Strain 2 in swine stated that Strain 2 was protective in swine which were challenged conjunctivally with virulent \textit{B. suis}, but offered no protection when vaccinated gilts were challenged with naturally infected boars. Strain 2 protection studies in mice found that Strain 2 induced equal protection of mice when challenged with virulent strains of \textit{B. suis, B. melitensis,} or \textit{B. abortus} 45 days after vaccination. The level of protection was similar when vaccinated mice were challenged 150 days after vaccination except for the virulent \textit{B. melitensis} challenge model in which there was no significant difference in total splenic weight or \textit{Brucella} recovery compared to nonvaccinated controls. Studies executed in Spain with Strain 2 failed to support the Chinese reports which stated that Strain 2 was efficacious in preventing ovine brucellosis.

With RB51 having showed great efficacy in controlling bison and cattle brucellosis, there has been great optimism for this vaccine to aid in the control of swine brucellosis in both feral and domestic populations. Initial studies with RB51 reported that it induced a humoral immune response in swine when administered parenterally or orally. Lord and colleagues evaluated the efficacy of RB51 in vaccinated gilts as compared to gilts vaccinated with killed \textit{B. suis} strain 1330 (biovar 1), extracts containing the O-side chain of the LPS from \textit{B. suis}, and non vaccinated controls. Oral and parenteral routes at various dosages were examined for all vaccine preparations. Single and multiple administrations of each vaccine were also examined. Lord concluded that oral and parenteral administration of either a single or multiple doses of both RB51 and the O-side chain vaccine preparation gave 100% protection in a natural challenge model utilizing naturally infected boars as the method of challenge for vaccinated gilts. Lord, however, did not evaluate culture recovery of \textit{Brucella} from all animals.
Bacteriologic culture was only performed on vaginal swabs and fetal tissues from gilts which aborted. Therefore, the criteria for protection for this study centered around protection from abortion. 

**Feral swine**

Little attention was paid to documenting the establishment, distribution, and propagation of feral swine during the early history of North America after European habitation; therefore, most of what has been written on the historical perspective of this animal is based on historical inference as well as oral tradition. Feral swine are believed to have existed in the territory which is now the continental United States since 1542 when the explorer Hernando DeSoto introduced swine into short-lived Spanish settlements. Feral swine on the Hawaiian islands are estimated to have originated with Polynesian bringing swine from Tahiti around 1000 AD. This population undoubtedly intermingled with European stock which was brought by Cook to the islands in 1778. Animals which are referred to as feral or wild swine are all members of the species *Sus scrofa* and consist of animals with at least 3 distinct phenotypes. The animal which is traditionally referred to as the feral hog or “razorback” is a descendent of those animals which went feral during the early stages of colonialism in North America. These early feral swine intermingled with “captive” swine which were free-range raised in the woodland and open areas of the southern US through the early part of the twentieth century. The Eurasian boar is the second phenotypically distinct wild pig in North America. These animals were imported from Europe first to the state of New Hampshire in the latter part of the nineteenth century. Established populations of “pure” Eurasian boars are recognized in New Hampshire and the southern Appalachian region of Tennessee and North Carolina. Domestic pigs which have been accidentally or intentionally released in recent years comprise the third phenotypic distinction. Of course, interbreeding among these three phenotypic groups exists.

Feral swine populations are established in at least 31 states in the US (Figure 1), and feral swine are the only mammalian species on the Federal Invasive Species List. This makes it illegal on a federal scale to
intentionally transport and release feral swine for the purpose of establishing a new population in an area which was previously free of feral swine. Despite this federal restriction, feral swine distribution continues to drastically increase. This increase is most often due to intentional release of wild swine for the purpose of establishing populations for hunting or to enhance animal numbers or characteristics which are desirable for “trophy” animals in preexisting populations. Additional factors which have contributed to the increased distribution and population of feral swine include: escape of wild swine from shooting preserves, natural movement and proliferation of established feral swine populations, abandonment of wild swine by agents unable to market them to shooting clubs, purposeful release of domestic swine for hunting, escape of domestic swine from confinement operations, and escape or abandonment of pet swine.

Feral swine impose numerous documented negative impacts on the environments which they inhabit. In the southern US, feral swine populations are extremely costly to the timber and pulpwood industries. After an area has been timbered, feral swine often move into new growth pine forests and feed on the delicate roots of the pine species. Feral swine have also been shown to virtually destroy the delicate bog environments of Hawaii. Ground nesting birds and other wildlife populations have been negatively impacted by feral swine predation, and native collared peccary populations which compete with the feral swine for food and territory in Texas and the Southeast are also negatively impacted by the increasing numbers of feral swine.

Documentation of brucellosis in feral swine populations has been almost solely based on serological surveys as well as anecdotal evidence based on outbreaks of brucellosis in domestic swine and cattle populations which had contact with feral swine. When *Brucella* serological reactors were necropsied and their tissue cultured, *B. suis* was identified. Through this data, brucellosis has been documented in 15 states within the US (Figure 1). Serological prevalence in infected feral swine populations ranges from 0.6%-53%. Feral swine have also been a source of *B. suis* infection for hunters and feral swine slaughterhouse workers in both the US and Australia. Feral swine also serve as a source of *Trichinella spiralis* and
pseudorabies virus, thus, threatening the US pseudorabies eradication effort.\textsuperscript{66,112}

Although brucellosis has not been documented in collared peccaries (\textit{Tayassu tajacu}) in the US, collared peccaries have been found to be infected with \textit{B. suis} by bacteriological culture and serology in South America.\textsuperscript{145}

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Figure 1. Feral swine and feral swine brucellosis within the United States. All states which contain their respective state abbreviations have documented feral swine populations. States shaded in red contain feral swine with documented brucellosis. States shaded in green contain feral swine, but there was no detectable brucellosis within those feral swine based on serological surveys. States shaded in yellow contain feral swine, but there have been no studies to document the presence of brucellosis within those populations. States shaded in white contain no known feral swine.
CHAPTER 3. DIAGNOSTIC CHARACTERIZATION OF A FERAL SWINE HERD ENZOOTICALLY INFECTED WITH BRUCELLA

A paper submitted to the Journal of Veterinary Diagnostic Investigation


Abstract

Eighty feral swine were trapped from a herd which had been documented to be seropositive for Brucella and which had been used for Brucella abortus RB51 vaccine trials on a 7,100 hectare tract of land in South Carolina. The animals were euthanized and complete necropsies were performed. Samples were taken for histopathology, Brucella culture, and Brucella serology. Brucella was cultured from 62 (77.5%) animals. Brucella suis was isolated from 55 animals (68.8%), and all isolates were biovar 1. Brucella abortus was isolated from 28 animals (35.0%), and isolates included field strain biovar 1 (21 animals; 26.3%), vaccine strain Brucella abortus S19 (8 animals, 10.0%), and vaccine strain Brucella abortus RB51 (6 animals, 7.5%). Males were significantly more likely to be culture positive than females (92.9% vs. 60.6%). Thirty-nine animals (48.8%) were seropositive. Males also had a significantly higher seropositivity rate than females (61.9% vs. 34.2%). The relative sensitivity rates were significantly higher for the standard tube test (44.6%) and fluorescence polarization assay (42.6%) than the card agglutination test (13.1%). Lesions consistent with Brucella infection were commonly found in the animals surveyed and included inflammatory lesions of the lymph nodes, liver, kidney, and male reproductive organs which ranged from lymphoplasmacytic to pyogranulomatous with necrosis. This is the first report of an apparent enzootic Brucella abortus infection in a feral swine herd suggesting that feral swine may serve as a reservoir of infection for Brucella abortus as well as Brucella suis for domestic livestock.

Introduction

The genus Brucella composes a group of genetically very similar, Gram negative, facultative intracellular, pathogenic bacteria which are known to infect over 80 domestic and wild mammalian species. In animal hosts, Brucellae commonly are
associated with infection of reproductive tissues of the male and female and lymphoreticular tissues as well as pyogranulomatous lesions of multiple tissues. Four members of the *Brucella* genus, *B. melitensis*, *B. suis*, *B. abortus*, and *B. canis* are zoonotic agents and produce a disease in humans referred to as undulant fever which is characterized by recurrent pyrexia, infection of lymphoreticular tissues, bacteremia, and purulent and pyogranulomatous lesions in multiple organs in infected humans. *B. melitensis*, *B. abortus*, and *B. suis* are also on the US Centers for Disease Control and USDA Select Agents list due to their perceived potential use as biological agents of terrorism and war.

The US has had a concerted effort to eliminate both cattle and swine brucellosis from its livestock populations through cooperative programs between the states and USDA Animal Plant Health Inspection Service (APHIS). Both programs have been based on the principle of serological testing and elimination of seroreactive herds. The eradication program in cattle has been aided by the implementation of calfhood vaccination with reduced virulence vaccine strains S19 and RB51. The swine program never implemented the use of a vaccine, but dramatic changes in swine management practices since the 1950s undoubtedly aided the elimination of swine brucellosis in domestic herds. With the near total eradication of brucellosis from the US livestock population, there is an increased effort to address the issue of *Brucella* infected wildlife populations. Within the 48 contiguous states of the US, feral swine (*Sus scrofa*), elk (*Cervus elaphus nelsoni*), and bison (*Bison bison*) are the major wildlife reservoirs of *Brucella*. Today only sporadic outbreaks of brucellosis occur in both domestic swine and cattle within the US, and most recent outbreaks have been associated with contact with *Brucella* infected feral swine or elk.

Within the US *B. abortus* is maintained enzootically within elk (*Cervus elaphus nelsoni*) and bison (*Bison bison*) within the Greater Yellowstone Area of Wyoming, Montana, and Idaho. Brucellosis has been reported in the bison of Yellowstone National Park since 1917, and current studies from the Greater Yellowstone Area report seroprevalence rates of 76-90% with 46% of seropositive females being culture positive. The bison herds have been managed to remain on federal land and likely have had
limited contact with cattle. However, elk numbers in the area are much higher. *Brucella* infected elk have a much larger geographic distribution than bison, and elk often come into close contact with cattle in the area. Recent brucellosis seroprevalence rates among elk herds associated with winter feed ground has been reported to be 25-54%.\(^7,18\) However, seroprevalence rates of elk herds not associated with feed ground has been reported to be 0-1%.\(^25\)

Feral swine herds have been reported in 27 of 50 states in the USA. The numbers and population distribution of feral swine have increased dramatically in recent decades partially due to natural population dynamics but mostly due to the establishment of new populations for the purpose of hunting in areas which were previously feral swine free. Brucellosis has been documented in feral swine populations in 14 states.\(^37\) In addition to the infection of domestic swine with *B. suis*, feral swine are also responsible for infecting cattle with *B. suis*.\(^10\) Due to their popularity as a game species and the widespread distribution of brucellosis within these populations, feral swine are also a significant threat for zoonotic infections.\(^28\)

**Materials and Methods**

This study was conducted on a 7,100 hectare tract of land on a peninsula between the Atlantic Ocean and the Winyah Bay in Georgetown County, South Carolina, USA (33° 20’ N, 79° 13’ W). The study was conducted between October 2002 and February 2003. A total of 80 sexually mature feral swine (42 males and 38 females) were trapped using box traps and fermented shelled corn as bait. This population had been used to evaluate the efficacy of parenteral administration of *B. abortus* RB51 (RB51) in feral swine.

**Necropsies:**

After euthanasia, 30 ml of blood was obtained via cardiac puncture. Blood was divided for serology (10 ml) and for *Brucella* culture (20 ml). Urine, nasal swabs, and vaginal swabs were collected for bacteriological culture. The following tissues were harvested for bacteriological culture and histologic examination: liver, spleen, lung, kidney, uterus, mammary tissue, testis, seminal vesicle, bulbourethral gland, prostate, and lymph nodes including prescapular, medial retropharyngeal, sternal, tracheobronchial,
gastrohepatic, prefemoral, popliteal, mandibular, and parotid. All samples for bacteriological evaluation were frozen at -70°C until processed for culture. Tissues collected for histologic evaluation were immediately placed in neutral-buffered 10% formalin, processed by routine paraffin embedding techniques, cut in 4 μm sections, and stained with hematoxylin and eosin. Selected tissue sections were also stained by Ziehl-Neelsen (acid-fast), periodic acid-Schiff (PAS), and Hucker-Twordt (silver) methods

**Serologic Evaluation**

After collection, blood samples were allowed to clot and serum was separated by centrifugation. Serum was divided into 1 ml aliquots, and stored at -70°C until assays were performed. *Brucella* serologic status of all animals was determined by fluorescence polarization (FPA)\(^2\), standard tube agglutination (STT)\(^1\), and card agglutination assays\(^1\) by previously described methods. Animals which had a positive reaction on at least one of the three serologic assays were considered positive seroreactors.

**Bacteriologic Culture:**

After thawing, tissues were individually ground in approximately 10% (w/v) sterile phosphate buffered saline (PBS, pH=7.2) using sterile glass grinders. Aliquots (100 μl) of each tissue homogenate were plated on tryptose agar containing 5% bovine serum as well as three *Brucella* selective media including brilliant green agar (BGB132; tryptose agar base with 5% bovine serum, .001 μg/ml brilliant green, 25 U/ml bacitracin, 100 μg/ml cycloheximide, 100 U/L nystatin, 20 μg/ml vancomycin, 50 μg/ml trimethoprim, and 100 μg/ml EDTA), Kudzas Morse agar (KM; tryptose agar base with 5% bovine serum, 25 U/ml bacitracin, 6 U/ml polymyxin B, 100 μg/ml cycloheximide, and 1.4 μg/ml ethyl violet), and RBM agar, a selective medium for SRB51\(^1\). Inoculated plates were incubated at 37°C in 5% CO\(_2\) for 7 days.

**Culture Identification:**

*Brucella* suspect cultures were identified on the basis of colony morphology, growth characteristics, and growth on selective media.\(^1\) Isolates were identified as *Brucella spp.* by a polymerase chain reaction technique (PCR) using *Brucella*-specific primers to the *omp2A* region of the *Brucella* genome. Reactions consisted of 50 μl and contained 5 μl of suspect culture in tris-EDTA and 45 μl of reaction mixture consisting of
200 μM each of dATP, dCTP, dGTP, and dTTP, 1 X PCR Buffer I\textsuperscript{b}, 1.5 mM MgCl\textsubscript{2}, 1.25 U AmpliTaq Gold polymerase\textsuperscript{b}, and 0.2 μM of each upstream and downstream primers (Table 1) selected from the omp2A sequences of \textit{B. abortus}.\textsuperscript{13} The primers had 100% conservancy within the genomes of \textit{B. suis} and \textit{B. melitensis} according to BLAST analysis. Tris-EDTA and a culture of \textit{Yersinia enterocolitica} O:9 served as negative controls, and \textit{B. suis} strain 3B, a biovar 1 laboratory challenge strain, served as a positive control. Following a 10 minute activation at 95°C, reaction preparations were cycled in a thermocycler\textsuperscript{c} for 40 cycles consisting of 30 sec. at 95°C, 30 sec. at 44°C, and 60 sec. at 72°C. Products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Animals were considered culture positive if at least one sample yielded a positive \textit{Brucella} culture.

Cultures which were positive by the \textit{omp2A} PCR assay were run in a second multiplex PCR which could discriminate \textit{B. suis}, \textit{B. abortus} RB51, \textit{B. abortus} S19, and \textit{B. abortus}/\textit{B. melitensis} field strains. This PCR assay utilized RB51 specific primers targeted toward the insertion sequence 711 (IS\textit{711}).\textsuperscript{4,32} Each reaction mixture consisted of a volume of 25 μl containing 2.5 μl of suspect culture in Tris-EDTA, 1X PCR reaction buffer containing 50 mM Tris, 1.5 mM MgCl\textsubscript{2}, 10 mM KCl, 50 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, pH=8.3\textsuperscript{d}, 200 μM each of dATP, dCTP, dGTP, and dTTP; 1X GC rich solution\textsuperscript{d}; 1.0 U of FastStart DNA polymerase\textsuperscript{c}; and 0.2 μM of the following primers: IS\textit{711} specific, RB51-3 , \textit{eri} multiplex forward, \textit{eri} multiplex reverse, 42797 forward, and 42797 reverse (Table 1). Thermocycling consisted of a single 5 min. incubation at 95°C followed by 40 cycles consisting of 15 sec. at 95°C, 30 sec. at 52°C, and 90 sec. at 72°C. Tris-EDTA and \textit{B. suis} strain 3B, SRB51, S19, and \textit{B. abortus} strain 2308 served as the positive control. Products were analyzed by electrophoresis on 2.0% agarose gels stained with ethidium bromide.

In order to determine the degree of genetic homogeneity among the feral swine isolates found in this study and to aid in the positive identification of \textit{B. abortus} vaccine strains, a variable nucleotide tandem repeat (VNTR) assay (“HOOF-Prints” assay) was employed as previously described.\textsuperscript{2,3} Isolates were assayed for the number of copies of
the 8 bp repeat (5’-AGGGCAGT-3’) at loci 1-8 as reported in the original paper describing the technique.³

**S19 Confirmation and Sequencing**

Isolates which were S19 based on the multiplex PCR were selected to determine if the deletion in the *eri* gene was the same as was previously reported by the reference strain of S19.²⁷ S19 isolates were subjected to a PCR reaction which flanked the deletion in the *eri* gene.²⁷ Each reaction consisted of 50μl and contained 5 μl of suspect culture in tris-EDTA and 45 μl of reaction mixture consisting of 200 μM each of dATP, dCTP, dGTP, and dTTP, 1 X PCR Buffer II, 1.5 mM MgCl₂, 1.25 U AmpliTaq Gold polymerase, and 0.2 μM of each eri sequencing forward and reverse primers (Table 1). Thermocycling consisted of a 10 minute activation at 95°C followed by 40 cycles consisting of 60 sec. at 95°C, 30 sec. at 57°C, and 30 sec. at 72°C. Laboratory propagated S19 and *B. abortus* strain 2308 served as positive controls and *Yersinia enterocolitica* O:9 served as a negative control. Products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. PCR products were purified in a silica matrix prior to sequencing. Products were quantitated using the Pico Green assay for dsDNA. The appropriate quantity of dsDNA was labeled in both directions using Big Dye terminator chemistries and sequenced using an ABI 3100 genetic analyzer. Primers used for sequencing were identical to those used in the primary PCR reaction and were used at a concentration of 0.13 μM. Resulting sequences were assembled and edited using Sequencher 3.0. Alignment of gene segments was performed with the program CLUSTALX.³¹

**Statistical Analysis**

For all statistical analyses a level of P<0.05 was used to determine differences between vaccinates and controls. Chi square analysis was used to determine differences between males and females within the population for serologic positivity and serologic sensitivity, differences among serology assays for sensitivity rates, and differences between culture positive and culture negative animals for histologic lesions which were not sex specific.
Fisher’s Exact Test was used to determine differences between males and females within the population for culture positivity, the isolation of multiple *Brucella* species, and the isolation of specific *Brucella* species as well as differences between culture positive and culture negative males for sex specific histologic lesions.

**Results**

**Necropsy findings and gross lesions**

Of the 38 females which were trapped, 15 (39.5%) were gestating and 7 (18.4%) were lactating. *In utero* litter size ranged from 2 to 7 (mean = 4.6; median = 5).

The seminal vesicles of one male were diffusely enlarged, fibrotic, and contained an abundant amount of purulent exudates. *B. suis* was subsequently cultured from the affected seminal vesicle. Three animals had grossly visible lymph node abscesses (2 gastrohepatic and 1 sternal). One animal had multiple pleural and mesenteric adhesions and abscesses. Five animals had multifocal, raised, firm hepatic nodules which were consistent with larval migrans tracts. Thirty-three animals had marked proliferation of the ureter and periureteral tissue which contained numerous adult nematodes consistent with *Stephanurus dentatus*.

**Histologic Lesions**

Histologic lesions are summarized in Table 2. There was no statistically significant correlation of any lesions with *Brucella* culture status. Five animals in the study had no significant lesions. Interstitial nephritis was the most common lesion found and was present in 37 animals. This ranged from multifocal to coalescing areas of lymphoplasmacytic infiltrates to large lymphofollicular aggregates in the renal interstitium (Figure 1A). Hepatitis was present in 34 animals. There were three general forms of hepatic inflammation: multifocal, random, lymphocytic hepatitis; multifocal, lymphoplasmacytic and purulent pericholangitis; and multifocal, random, pyogranulomatous hepatitis with necrosis (Figure 1B). Some of the pyogranulomas were associated with nematode larvae. Lymphadenitis was present in 29 animals. The lymphadenitis was often multicentric and ranged from diffuse, purulent lymphadenitis to multifocal pyogranulomas often with necrotic cores (Figure 1C).
Histologic lesions within male reproductive organs were common findings. Orchitis was present in 11 males and was characterized by multifocal to diffuse lymphocytic (Figure 1D) or multifocal to diffuse granulomatous inflammatory infiltrates. All cases of orchitis were associated with significant seminiferous tubular atrophy. Seminal vessiculitis was observed in 6 males and was exhibited in 3 patterns: multifocal, lymphoplasmacytic seminal vessiculitis; diffuse, purulent and lymphoplasmacytic seminal vessiculitis with fibrosis (Figure 1E); and diffuse, granulomatous seminal vessiculitis with marked fibrosis. Multifocal lymphocytic prostatitis was also present in 3 males (Figure 1F).

Multifocal renal granulomas were present in 2 animals. Marked epididymal fibrosis was present in 2 males. One animal exhibited multifocal-coalescing, purulent splenitis, and one female exhibited diffuse purulent endometritis. No pyogranulomatous or granulomatous lesions in any tissues were associated with fungi or acid-fast bacteria.

Lesions which were not consistent with swine brucellosis were also present. Thirty-four animals had eosinophilic and granulomatous ureteritis and periureteritis with intralesional trichostrongyles consistent with *Stephanurus dentatus*. Moderate to marked interstitial cell hyperplasia was present in the testes of 21 males. Eleven animals exhibited eosinophilic and granulomatous interstitial pneumonia with intralesional metastrongyles, and one animal had amyloidosis of the liver, kidney, and spleen.

**Bacteriologic Culture**

*Brucella* was isolated from a total of 62 (77.5%) feral swine in this study. A multiplex PCR which could distinguish among *B. suis*, *B. abortus* field strains, S19, and RB51 was used in these studies because of the history of using RB51 in this population for vaccine studies. The multiplex PCR assay revealed that not only was *B. suis* and RB51 present in the population but also *B. abortus* field strains and S19 were present (Figure 2). The results of the multiplex PCR assay correlated 100% with traditional *Brucella* biotyping methods. All *B. suis* isolates were biovar 1 and all *B. abortus* field strain isolates were also biovar 1. Table 3 lists the species of *Brucella* isolated on a per animal basis. Isolation of *B. suis* only was the most common finding; however, multiple species were isolated from 21 animals (26.3% of the total population; 33.9% of the
culture positive animals). *B. suis* was isolated from 55 animals (68.8% of the total population; 88.7% of the culture positive animals). *B. abortus* was isolated from 28 animals (35.0% of the total population; 45.2% of the culture positive population). Of these 28 *B. abortus* positive animals, biovar 1 field strains were isolated from 21 animals (26.3% of the total population; 33.9% of the culture positive population); S19 was isolated from 8 animals (10.0% of the total population; 12.9% of the culture positive population); and RB51 was isolated from 6 animals (7.5% of the total population; 9.7% of the culture positive population). Table 4 lists the number of isolates made for the various species on a per tissue basis. In general there tended to be a dominant species in each tissue even in animals which were infected with multiple tissues. However, isolation of multiple species of *Brucella* in particular tissues was achieved.

Males were significantly more likely to be *Brucella* culture positive than females (P<0.001). The culture positivity rate was 92.9% (39/42) for males and 60.5% (23/38) for females. There was no statistically significant difference between males and females in the culture positivity rates for any individual species of *Brucella* found in this study when the results were compared among culture positive animals only or among the entire population. Among culture positive animals, females were more often infected with multiple *Brucella* species than males (60.9% for females vs. 20.5% for males; P<0.01); however, there was no significant difference when the data was compared among the entire population.

The HOOF-Prints (VNTR) assay suggested that the *B. suis* isolates were not highly heterogeneous (Table 5). The number of alleles was conserved in the entire group of isolates at loci 2, 3, and 4; however, differences were found at all other loci. The isolates from a given animal tended to have identical patterns; however, occasionally patterns from isolates from different tissues from the same animal had different patterns. This is evidenced in isolates pairs 1688-3/1690-1 and 1803-1/1819-1 in table 5. Selected VNTR patterns for *B. abortus* isolates are given in Table 6. All *B. abortus* biovar 1 field strains had conserved patterns at loci 1, 2, and 3; however there were single or double allelic differences at all other loci. All RB51 isolated from feral swine had identical patterns as the laboratory reference strain of RB51. Most S19 feral swine
isolates had identical patterns to the laboratory reference strain of S19; however, 2 isolates had VNTR patterns which differed from the S19 laboratory reference strain. The isolate 7-pop (Table 6) differed by 3 alleles at locus 3 from the S19 reference strain, and the isolate 1665-1 differed from the S19 reference strain at loci 1, 6, and 7 by 1, 1, and 4 alleles respectively.

**S19 Sequencing**

Four isolates which typed as S19 by the multiplex PCR assay and by traditional biotyping were selected for sequencing of the *eri* gene in order to determine if the deletion within the *eri* gene matched the laboratory reference strain of S19. All 4 isolates and the S19 laboratory strain yielded the expected PCR product of approximately 361 bp. The resulting sequences from all 4 of the feral swine isolates were 100% homologous to the sequence obtained from the S19 laboratory strain.

**Serology**

The results of the serologic assays are listed in Table 7. The seropositivity rates were 48.8% for the entire population, 61.9% for males, and 34.2% for females. Males were significantly more likely to be seropositive among the entire population (P<0.05); however, there was no difference in seropositivity rates between males and females among culture positive animals.

Relative sensitivity rates for each assay and a combination of the three assays are also listed in Table 7. To estimate the sensitivity the number of seropositive animals from the culture positive group was divided by the number of culture positive animals. One animal yielded only RB51 on bacteriologic culture, and that animal was excluded from the sensitivity estimates as RB51, being O-polysaccharide deficient, does not induce detectable immune responses by the three assays utilized in this study.\(^{21,29}\) The sensitivity rates of both the STT (P<0.0001) and FPA (P<0.005) assays were significantly higher than the Card Test but did not differ from each other.

**Discussion**

In this study 80 feral swine from an area of enzootic feral swine brucellosis on the Atlantic coast of South Carolina were trapped, euthanized, and necropsied to determine *Brucella* culture, serologic, and lesion profiles. It was found that not only were the feral
swine in this herd infected with biovar 1 of *B. suis* but also with biovar 1 field strains of *B. abortus* and the vaccine strains *B. abortus* S19 and *B. abortus* RB51. This is the first report of *B. abortus* in feral swine and the first report of a wildlife reservoir of *B. abortus* outside the Greater Yellowstone National Park Area of Wyoming, Montana, and Idaho.

The finding of well established *B. abortus* infection in this feral swine herd was surprising not only because it had never been reported but also due to experimental evidence which shows that swine become only briefly infected with *B. abortus* when experimentally challenged with *B. abortus*. Previous studies in domestic swine have shown that RB51 is cleared quickly and persists only to four weeks in lymph nodes draining the site of infection. In the current study, however, *B. abortus* was found in a variety of tissues including whole blood suggesting that bacteremia and systemic distribution of the *B. abortus* occurs in feral swine.

The introduction of *B. abortus* S19 and biovar 1 field strains in this feral swine herd was possibly due to scavenging of aborted fetuses of dead, *B. abortus* infected cattle at a time when cattle were kept on the same property or in the vicinity. The introduction of RB51 is easily explained by the use of this herd in experiments evaluating the potential usage of this vaccine in feral swine. However, no such experiments were ever conducted in this herd with the S19 vaccine strain. Examination of property records suggests the possibility of longstanding *B. abortus* infection in this feral swine herd. No cattle have been kept in the area of the feral swine since at least 1970. The property is bordered by the Atlantic Ocean, the Winyah Bay, and a coastal housing development and country club which greatly limit the migration of feral swine into and out of the area. The VNTR data supported long standing infections of *B. suis*, *B. abortus* biovar 1 field strain, and *B. abortus* S19 since these isolates had allelic differences at multiple loci. However, all RB51 isolates examined showed the same pattern as the reference strain of RB51.

The *Brucella* culture positivity rate in this feral swine herd was high which reflects the tendency of swine to develop chronic *Brucella* infections. The serologic sensitivity rates were much lower than those previously reported for the assays used in this study. The results of this study serve as an example for the lack of sensitivity of serological assays for diagnosing brucellosis in individual feral swine.
culture results were compared to necropsy serology results, the sensitivity of a combination of the three serological assays which were used was 54.1%. Previously published sensitivity rates for these assays in domestic swine were 20-67% for the card test, 83-100% for the standard tube test, and 80-94% for the FPA test.\textsuperscript{12,19,22} The sensitivity rates for individual assays calculated in this study, however, were 13.1% for the card test, 44.6% for the standard tube test, and 42.6% for the fluorescence polarization assay. Antigens used in these assays are from \textit{B. abortus} which may at least partially account for the lack of sensitivity when evaluating swine presumably infected with \textit{B. suis}. The lack of sensitivity may also be reflected in the chronicity of infection in the animals in this study. Samples used to determine sensitivity rates in previously published reports were obtained from acute outbreaks of swine brucellosis. The relative sensitivity rates of these serologic assays have not been previously investigated in enzootically infected herds. Antibody decay may occur despite the persistence of culturable bacteria in tissues.

This study utilized a multiplex PCR assay for the identification of \textit{Brucella} species. The assay could definitively identify \textit{B. suis}, \textit{B. abortus} RB51, and \textit{B. abortus} S19. The assay could also identify \textit{Brucella} species other than \textit{B. suis} and the two \textit{B. abortus} vaccine strains; however, it could not differentiate among the other species. Within this assay, a single primer pair was utilized to differentiate between \textit{B. suis} and other \textit{Brucella} species (\textit{B. abortus} and \textit{B. melitensis}). These primers (42797 forward and reverse) were targeted to a location on chromosome II at which \textit{B. suis} has a 189 bp insertion. This size difference allowed easy discrimination of \textit{B. suis} from the other \textit{Brucella} species on agarose gels. Bricker has previously reported on the use of multiplex PCR (AMOS assay) to discriminate \textit{Brucella} species.\textsuperscript{4,5} The AMOS assay utilized species differences in the IS711 insertion sequence and required individual primers for species identification.\textsuperscript{4} Fayazi and colleagues have also reported on the use of a PCR assay with a single primer pair which can distinguish \textit{B. suis} from \textit{B. abortus}.\textsuperscript{11} The primer pairs used by Fayazi were unsuccessful in identifying either \textit{Brucella} species in the studies reported in this paper. Upon examination of the genetic sequence used to design Fayazi’s primers, it was found that the sequence was an assembly of 4 unique,
nonlinked MboI fragments. The corresponding sequences are as follows: 1) Nucleotides 1-149 of the Fayzi sequence align to nucleotides 1595585-1595734 of chromosome I of B. abortus 9-941; 2) nucleotides 146-200 of the Fayazi sequence align to nucleotides 225993-225939 of chromosome I of Brucella abortus; 3) Nucleotides 195-330 of the Fayazi sequence align to nucleotides 582796-582668 of Chromosome II B. abortus; and 4) Nucleotides 336-482 of the Fayazi sequence align to nucleotides 53279-538430 of chromosome II of B. abortus.16 The forward primer used in Fayazi’s assay corresponds to positions 1595587-1595611 of chromosome I of B. abortus, and the reverse primer corresponds to positions 538397-538421 of chromosome II of B. abortus. In light of the sequence data, it is easily explained that the primers used in the Fayazi PCR assay could not identify either Brucella species in the current study. It also appears that the current study is the first report of a single primer pair which can distinguish B. suis from other Brucella species.

Animals within this herd had histologic lesions in multiple organs which were consistent with swine brucellosis20; however, none of these lesions could be significantly correlated to Brucella infection within the animal or within the tissue containing the lesion. Despite a high culture positivity rate, the number of animals with reproductive lesions was surprisingly low. Of the 39 culture positive males in this study, only 14 had lesions in reproductive tissues, and of the 23 culture positive females in this study, only one exhibited endometritis and 2 exhibited placental necrosis. In the face of a high level of Brucella within this population, these feral swine manage to maintain a level of reproductive efficiency which supports significant population growth and warrants regular population control strategies in order to control the amount of environmental damage imposed by the feral swine herd.

The results of this study have identified a feral swine herd which is enzootically infected with B. suis as well as multiple strains of B. abortus. This study shows that within an enzootically infected feral swine population, the seroprevalance rates may be significantly lower than the true infection rate. The results of this study also reiterate the possibility of feral swine to serve as reservoirs of Brucella spp. for both domestic livestock and human infections.
Acknowledgements

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Names are necessary to report factually on available data. Mention of trade names or commercial products in the article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture.

References


35. Williams ES, Cain SL, Davis DS: 1997, Brucellosis: the disease in bison In: Brucellosis, bison, elk, and cattle in the Greater Yellowstone Area: defining the problem, exploring solutions. Thorne Et, Boyce MS, Nicoletti P, Kreeger TJ eds. pg. 219 Wyoming Game and Fish Department, Cheyenne, WY.


Sources and Manufacturers

aBoehringer Mannheim, Indianapolis, IN, USA.

bPerkin Elmer, Branchburg, NJ, USA.

cMJ Research Inc., Watertown, MA, USA.

dRoche Molecular Biochemicals, Indianapolis, IN, USA.

eRoche Diagnostics, Basel, Switzerland.

fGeneClean II kit, Q-Biogene, Irvine, CA, USA.

gMolecular Probes, Eugene, OR, USA.

hApplied Biosystems Inc., Foster City, CA, USA.

iGene Codes Corp., Ann Arbor, MI, USA.

jPROC FREQ CHISQ, SAS Statistical Software, SAS Institute, Inc., Cary, NC, USA.
Table 1. Primer Sequences used in PCR and sequencing assays for the identification of *Brucella* isolates obtained from feral swine.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide Sequence 5’-3’</th>
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<tr>
<td><em>Omp2A</em> Forward</td>
<td>GCAACGGGTGTTCTTCCACTC</td>
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<tr>
<td><em>Omp2A</em> Reverse</td>
<td>GTATCAGGCTACGCAGAAGG</td>
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<td>IS711 specific</td>
<td>TGCCGATCCTAAGGGCCTTCATTGCCAG</td>
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<td>RB51-3</td>
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<tr>
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<tr>
<td><em>eri</em> Multiplex Reverse</td>
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</tr>
<tr>
<td>42797 Forward</td>
<td>CGATGTGCTGGCGCGAAACCTTGTAC</td>
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<tr>
<td>42797 Reverse</td>
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<td>TTGGCGGCAAGTCCGTCGGT</td>
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<tr>
<td><em>eri</em> Sequencing Reverse</td>
<td>CCCAGAAGCGGACGAAACG</td>
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Table 2. Summary of histologic lesions observed in 80 feral swine (42 males, 38 females) trapped from a herd enzootically infected with *Brucella* spp.

<table>
<thead>
<tr>
<th>Lesions Consistent with <em>Brucella</em> infection</th>
<th>Total # Animals</th>
<th>Culture a</th>
<th>Organ b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interstitial Nephritis c</td>
<td>37 (46.3%)</td>
<td>29 (78.4%)</td>
<td>1 (2.7%)</td>
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<tr>
<td>Hepatitis d</td>
<td>34 (42.5%)</td>
<td>29 (85.3%)</td>
<td>7 (20.6%)</td>
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<td>Lymphadenitis e</td>
<td>29 (36.3%)</td>
<td>25 (86.2%)</td>
<td>4 (13.8%)</td>
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<td>Orchitis f</td>
<td>11 (26.2%)</td>
<td>11 (100%)</td>
<td>5 (45.5%)</td>
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<tr>
<td>Seminal Vessiculitis g</td>
<td>6 (14.3%)</td>
<td>6 (100%)</td>
<td>3 (50.0%)</td>
</tr>
<tr>
<td>Prostatitis h</td>
<td>3 (7.1%)</td>
<td>2 (66.7%)</td>
<td>0</td>
</tr>
<tr>
<td>Renal Granulomas</td>
<td>2 (2.5%)</td>
<td>2 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Epididymal fibrosis</td>
<td>2 (2.5%)</td>
<td>2 (100%)</td>
<td>1 (50.0%)</td>
</tr>
<tr>
<td>Placental necrosis</td>
<td>2 (2.5%)</td>
<td>1 (50.0%)</td>
<td>0</td>
</tr>
<tr>
<td>Splenitis, purulent</td>
<td>1 (1.3%)</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Endometritis, purulent</td>
<td>1 (1.3%)</td>
<td>1 (100%)</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lesions not consistent with <em>Brucella</em> infection</th>
<th>Total # Animals</th>
<th>Culture a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulomatous ureteritis i</td>
<td>34 (42.5%)</td>
<td></td>
</tr>
<tr>
<td>Interstitial cell hyperplasia</td>
<td>21 (50.0%)</td>
<td></td>
</tr>
<tr>
<td>Verminous pneumonia i</td>
<td>11 (13.8%)</td>
<td></td>
</tr>
<tr>
<td>Multicentric Amyloidosis</td>
<td>1 (1.3%)</td>
<td></td>
</tr>
</tbody>
</table>

a Number of animals with the lesion which were culture + for Brucella.

b Number of animals with the lesion which were culture + for Brucella in the organ with the lesion.

c Multifocal, lymphoplasmacytic to lymphofollicular interstitial nephritis.

d Lesions included multifocal, random, lymphocytic hepatitis; multifocal, lymphoplasmacytic and purulent pericholangitis; and multifocal, random, pyogranulomatous hepatitis with necrosis.

e Lesions included diffuse, purulent lymphadenitis and multifocal, pyogranulomatous lymphadenitis with necrosis.

f Lesions included multifocal to diffuse lymphocytic orchitis and multifocal to diffuse granulomatous orchitis; both included seminiferous tubular atrophy.

g Lesions included multifocal, lymphoplasmacytic seminal vesciculitis; diffuse, purulent and lymphoplasmacytic seminal vesciculitis with fibrosis; and diffuse, granulomatous seminal vesciculitis with marked fibrosis.

h Multifocal, lymphocytic, prostatitis.

i Diffuse, granulomatous and eosinophilic, ureteritis with intralesional trichostrongyles consistent with *Stephanurus dentatus*.

j Multifocal, granulomatous and eosinophilic interstitial pneumonia with intralesional metastrongyles.
Table 3. Bacteriologic culture results. The species of Brucella obtained from 80 feral swine is given on a per animal basis.

<table>
<thead>
<tr>
<th>Brucella species isolated</th>
<th>Total culture positive feral swine&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. suis&lt;sup&gt;b&lt;/sup&gt; only</td>
<td>34 (42.5%, 54.8%)</td>
</tr>
<tr>
<td>B. abortus&lt;sup&gt;c&lt;/sup&gt; field strain only</td>
<td>5 (6.3%, 8.1%)</td>
</tr>
<tr>
<td>B. abortus RB51 (RB51) only</td>
<td>1 (1.3%, 1.6%)</td>
</tr>
<tr>
<td>B. suis and B. abortus field strain</td>
<td>11 (13.8%, 17.7%)</td>
</tr>
<tr>
<td>B. suis and B. abortus S19 (S19)</td>
<td>4 (5.0%, 6.5%)</td>
</tr>
<tr>
<td>B. suis and B. abortus RB51</td>
<td>2 (2.5%, 3.2%)</td>
</tr>
<tr>
<td>B. abortus field strain, S19, and RB51</td>
<td>1 (1.3%, 1.6%)</td>
</tr>
<tr>
<td>B. suis, B. abortus field strain, and RB51</td>
<td>1 (1.3%, 1.6%)</td>
</tr>
<tr>
<td>B. suis, B. abortus field strain, and S19</td>
<td>2 (2.5%, 3.2%)</td>
</tr>
<tr>
<td>B. suis, B. abortus field strain, S19, and RB51</td>
<td>1 (1.3%, 1.6%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>A total of 80 feral swine were trapped, and a total of 62 (77.5%) were culture positive for at least one species of Brucella. The number reported is the total number of feral swine positive for the respective species of Brucella with the percentage of the total population followed by the percentage of culture positive animals in parentheses.

<sup>b</sup>All B. suis isolates were biovar 1.

<sup>c</sup>All B. abortus field strain isolates were biovar 1.
Table 4. *Brucella* culture results by tissue. The results of bacteriologic culture of tissues from 80 feral swine is shown on a per tissue basis as well as the number of each species isolated for each given tissue.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total(^a)</th>
<th>B. suis(^b)</th>
<th>B. abortus field strain(^c)</th>
<th>B. abortus S19</th>
<th>B. abortus RB51</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph nodes</td>
<td>38 (61.3%)</td>
<td>26</td>
<td>11</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Popliteal</td>
<td>18 (22.5%)</td>
<td>12</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Tracheobronchial</td>
<td>16 (20.0%)</td>
<td>11</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Sternal</td>
<td>14 (17.5%)</td>
<td>9</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Gastrohepatic</td>
<td>13 (16.3%)</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mandibular</td>
<td>13 (16.3%)</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Prescapular</td>
<td>13 (16.3%)</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Parotid</td>
<td>13 (16.3%)</td>
<td>9</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Prefemoral</td>
<td>12 (15.0%)</td>
<td>9</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Medial</td>
<td>12 (15.0%)</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Male reproductive organs</td>
<td>22 (56.4%)</td>
<td>17</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Seminal Vessicle(^d)</td>
<td>16 (38.1%)</td>
<td>12</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Bulbourethral gland(^d)</td>
<td>13 (31.0%)</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Prostate(^d)</td>
<td>12 (28.6%)</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Testis(^d)</td>
<td>8 (19.1%)</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Nasal Swab</td>
<td>27 (33.8%)</td>
<td>20</td>
<td>5</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Blood</td>
<td>25 (31.3%)</td>
<td>24</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Urine</td>
<td>15 (18.8%)</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vaginal Swab(^e)</td>
<td>7 (18.4%)</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Liver</td>
<td>13 (16.3%)</td>
<td>9</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>13 (16.3%)</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Mammary(^e)</td>
<td>6 (15.8%)</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>12 (15.0%)</td>
<td>9</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Uterus(^e)</td>
<td>4 (10.5%)</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>6 (7.5%)</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)A total of 80 feral swine were trapped, and a total of 62 (77.5%) were culture positive for at least one species of *Brucella*.
\(^b\)All *B. suis* isolates were biovar 1.
\(^c\)All *B. abortus* field strain isolates were biovar 1.
\(^d\)There were 42 total males; 39 (60.6%) were *Brucella* culture +.
\(^e\)There were 38 total females; 23 (92.9%) were *Brucella* culture +.
Table 5. VNTR Patterns of Selected *B. suis* biovar 1 isolates from feral swine.

<table>
<thead>
<tr>
<th>Isolate or Strain Designation</th>
<th>VNTR-1</th>
<th>VNTR-2</th>
<th>VNTR-3</th>
<th>VNTR-4</th>
<th>VNTR-5</th>
<th>VNTR-6</th>
<th>VNTR-7</th>
<th>VNTR-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1330 (biovar 1 reference)</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>8</td>
<td>5</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>686 (biovar 3 reference)</td>
<td>8</td>
<td>4</td>
<td>10</td>
<td>2</td>
<td>10</td>
<td>6</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>760-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>782-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>1688-3&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>1714-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>7</td>
<td>4</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>344-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>401-1</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>9</td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>1690-1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>8</td>
<td>4</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>353-1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>9</td>
<td>3</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>389-1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>9</td>
<td>3</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>1857-1</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>9</td>
<td>3</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>1803-1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>1819-1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>8</td>
<td>4</td>
<td>7</td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Identical pattern  
<sup>b</sup>Differs from pattern a by 1 allele at a single locus  
<sup>c</sup>Isolates from the same animal (#52). 1688-3 isolated from the popliteal lymph node. 1690-1 isolated from the bulbourethral gland  
<sup>d</sup>Identical pattern  
<sup>e</sup>Isolates from the same animal (#216). 1803-1 isolated from the liver. 1819-1 isolated from the seminal vesicle
Table 6. VNTR Patterns of Selected *B. abortus* isolates from feral swine.

<table>
<thead>
<tr>
<th>Isolate or Strain Designation</th>
<th>VNTR-1</th>
<th>VNTR-2</th>
<th>VNTR-3</th>
<th>VNTR-4</th>
<th>VNTR-5</th>
<th>VNTR-6</th>
<th>VNTR-7</th>
<th>VNTR-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>544&lt;sup&gt;a&lt;/sup&gt; (biovar 1 reference strain)</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>9-941&lt;sup&gt;a&lt;/sup&gt; (<em>B. abortus</em> sequence strain)</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>399-1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>400-1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>402-1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>431-1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>RB51&lt;sup&gt;ai&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>1671-1&lt;sup&gt;ii&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>S19&lt;sup&gt;g&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>1678-1&lt;sup&gt;ij&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>1679&lt;sup&gt;ij&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>1684&lt;sup&gt;ij&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>7-pop&lt;sup&gt;cgi&lt;/sup&gt;</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>1665-1&lt;sup&gt;cgi&lt;/sup&gt;</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>12</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Laboratory reference culture.
<sup>b</sup>Isolates from the same animal (#10). Isolate 399-1 was isolated from the kidney. Isolate 400-1 was isolated from the liver, and 401-1 was isolated from the mandibular lymph node.
<sup>c</sup>Isolates are *B. abortus* biovar 1 field strains.
<sup>d</sup>*B. abortus* RB51.
<sup>e</sup>*B. abortus* S19.
<sup>f</sup>Isolates from the same animal (#50). Isolate 1678-1 was isolated from the liver, and 1679 was isolated from the mammary.
<sup>g</sup>Isolates from the same animal (#7). Isolate 7-pop was isolated from the popliteal lymph node, and 1665-1 was isolated from the prostate.
<sup>h</sup>Identical patterns.
<sup>i</sup>Identical patterns.
<sup>j</sup>Identical patterns.
Table 7. Serologic Results. Results of serologic assays from necropsy sera from 80 feral swine.

<table>
<thead>
<tr>
<th>Test</th>
<th>Total Seropositive</th>
<th>Total Seropositive from Culture Positive Group&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39/80 (48.8%)</td>
<td>33/61 (54.1%)</td>
</tr>
<tr>
<td>Card Test</td>
<td>9/80 (11.3%)</td>
<td>8/61 (13.1%)</td>
</tr>
<tr>
<td>Standard Tube Test</td>
<td>31/80 (38.8%)</td>
<td>27/61 (44.6%)</td>
</tr>
<tr>
<td>Fluorescence Polarization Assay</td>
<td>30/80 (37.5%)</td>
<td>26/61 (42.6%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>There were 62 culture positive animals; however, from one animal only RB51 was isolated. Because RB51 does not induce a serologic response which can be detected by the assays used, that animal was excluded from the sensitivity calculations.

<sup>b</sup>An animal was considered seropositive if there was a positive reaction on at least one serologic assay examined.
Figure 1A. Lymphofollicular interstitial nephritis. The renal interstitium is markedly expanded due to abundant numbers of lymphocytes which are organized into follicle-like structures. HE.

Figure 1B. Liver microgranulomas. The Hepatic parenchyma is replaced by multiple granulomas with necrotic centers. The area between the microgranulomas contains abundant collagen as well as scattered lymphocytes and plasma cells. HE.
Figure 1C. Lymph node, poorly organized pyogranuloma. Abundant neutrophils are surrounded by macrophages and lymphocytes as well as loosely organized fibrous tissue. Numerous multinucleated giant cells also segmentally surround the pyogranuloma. HE.

Figure 1D. Lymphocytic orchitis with atrophy of the seminiferous tubules. Seminiferous tubules are greatly separated from one another due to abundant numbers of small lymphocytes. The remaining seminiferous tubules are completely devoid of maturing spermatogonia; only Sertoli cells remain within the seminiferous tubules. HE.
Figure 1E. Purulent and lymphoplasmacytic seminal vesiculitis with fibrosis. Periglandular interstitial areas are expanded due to abundant numbers of lymphocytes and plasma cells as well as fibrous tissue. The glandular lumena contain numerous neutrophils and are lacking normal secretory fluid. HE.

Figure 1F. Lymphocytic prostatitis. Periglandular interstitial areas contain abundant numbers of small lymphocytes. HE.
Figure 2. 2% agarose ethidium bromide stained gel containing products from the multiplex PCR reactions from feral swine *Brucella* isolates. The lanes contain the following: 1. 100 bp ladder, 2. Negative control, 3-8. Feral swine *Brucella* isolates, 9. S19 positive control, 10. *B. abortus* 2308 positive control, 11. RB51 positive control, 12. *B. suis* 3B positive control.
CHAPTER 4. RESULTS OF A VACCINE TRIAL USING *BRUCELLA ABORTUS* RB51 IN A FERAL SWINE HERD ENZOOTICALLY INFECTED WITH *BRUCELLA*

A paper submitted to Research in Veterinary Science

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Abstract

The objective of this study was to evaluate the effect on transmission of *Brucella* and clearance of *Brucella abortus* strain RB51 (SRB51) vaccine in a feral swine herd with enzootic brucellosis. Feral swine (*Sus scrofa*) were trapped and injected intramuscularly with either 2 x 10^{10} CFU (2 ml) SRB51 (n=122) or 2 ml of saline (n=122). At the time of initial trapping blood was obtained to determine serologic status, and the animals were identified by eartags and microchip implants prior to being released. Ten to 14 months after initial capture 80 animals were recaptured. Animals were euthanized and necropsies were performed. Samples to determine serologic status and *Brucella* culture status were collected at the time of necropsy. There was no significant difference observed in seroconversion or in *Brucella* culture positivity rate between vaccinates and nonvaccinates. Also, there was no significant difference in anti-SRB51 dot blot titers between vaccinates and controls. SRB51 was isolated from necropsy samples from eight of forty-four vaccinated animals. The results of this study indicate that parenteral *Brucella abortus* strain RB51 has no effect in preventing transmission of *Brucella* within an enzootically infected feral swine herd.

Introduction

Feral swine herds have been reported in 26 of 50 states in the USA (Gipson, 1999; Gipson *et al.*, 1999). With increasing popularity of these animals as a game species, the geographic distribution and number of these populations are increasing. Brucellosis has
been documented in feral swine populations in 14 states (Zygmont et al., 1982). The transmission of Brucella from feral swine to domestic livestock has also been documented (Ewalt et al., 1997). With brucellosis nearly eradicated from domestic livestock in the USA, recent attention has been given to dealing with wildlife reservoirs, including feral swine. Currently, due to hunting and trapping and processing of these animals for the “wild boar meat” market, feral swine serve as a source of infection for human brucellosis in the USA (Starnes et al., 2004). Particular interest has developed in determining control strategies for brucellosis within wild swine populations in order to prevent further outbreaks of this disease in domestic livestock and humans.

Brucella abortus strain RB51 (SRB51) is a laboratory derived lipopolysaccharide O-side chain-deficient mutant (Schurig et al., 1991) of B. abortus strain 2308. SRB51 induces protective immunologic responses in cattle and bison against challenge-exposure with virulent B. abortus strains (Cheville et al., 1993; Cheville et al., 1996; Olsen et al., 2003). Initial reports suggested SRB51 induces an immune response (Edmonds et al., 2001) and prevents transmission of B. suis in swine (Lord et al., 1998). However, this has never been investigated in an enzootically infected feral swine population.

This study was designed to characterize the effect of SRB51 vaccination in preventing Brucella transmission in a feral swine population with a high prevalence of brucellosis; determine if serologic status influences efficacy of SRB51 under field conditions; and determine if feral swine stay persistently infected with RB51 after vaccination.

Materials and Methods

This study was conducted on a 7,100 hectare tract of land on a peninsula between the Atlantic Ocean and the Winyah Bay in Georgetown County, South Carolina, USA (33° 20’ N, 79° 13’ W). The study was conducted between September 2001 and February 2003.

Trapping and Vaccination of Feral Swine

Feral swine were trapped in wooden box traps with drop doors which were baited with fermented shelled corn. Initial trapping occurred weekly between September 2001
and May 2002. Upon trapping animals were snared and 10 ml of blood was obtained by cranial vena caval puncture for serologic assays. Plastic ear tags were placed in each ear and a digital identification chip (Digital Angel, South Saint Paul, MN) was placed subcutaneously at the base of one ear. Animals were injected with either 2.0 ml of a suspension containing approximately $2 \times 10^{10}$ CFU of freshly reconstituted lyophilized SRB51 (n=122) (Colorado Serum Company, Denver, CO, USA) or 2.0 ml saline (n=122). Adult and postweaning juvenile swine were utilized. Juvenile swine which were nursing were excluded due to possible passive maternal antibodies against *Brucella* spp.

Between October 2002 and February 2003, as many of the 244 original animals as possible were recaptured. A total of 80 feral swine from the original group of 244 were recaptured over a total of 20 individual days of trapping. These animals were euthanized and necropsied.

**Necropsies**

After euthanasia, 30 ml of blood was obtained via cardiac puncture. Blood was divided for serology (10 ml) and for *Brucella* culture (20 ml). The following tissues, fluids, and swabs were harvested for bacteriological culture: liver, spleen, lung, kidney, uterus, mammary tissue, testis, seminal vesicle, bulbourethral gland, prostate, urine, nasal swab, vaginal swab, and lymph nodes including prescapular, medial retropharyngeal, sternal, tracheobronchial, gastrohepatic, prefemoral, popliteal, mandibular, and parotid. All tissues were frozen at -70° C until processed for bacteriological evaluation.

**Serologic Evaluation**

After collection, blood samples were allowed to clot and serum was separated by centrifugation. Serum was divided into 1 ml aliquots, and stored at -70° C until assays were performed. *Brucella* serologic status of all animals was determined by fluorescence polarization (Pauolo *et al*., 2000), standard tube agglutination, and card agglutination assays (Alton *et al*., 1988) by previously described methods. Animals which had a positive reaction on at least one of the three serologic assays were considered positive seroreactors. Serological titers to SRB51 were determined in the 80 recaptured animals by a previously described dot blot assay (Olsen *et al*., 1997) in which $\gamma$-irradiated SRB51
was used as the antigen and peroxidase-labeled rabbit anti swine IgG (Sigma, St. Louis, MO, USA) was used as the secondary antibody at a dilution of 1:500.

**Bacteriologic Culture**

After thawing, tissues were individually ground in approximately 10% (w/v) sterile PBS (pH=7.2) using sterile glass grinders. Aliquots (100 μl) of each tissue homogenate were plated on 4 *Brucella* selective media. Media included tryptose agar containing 5% bovine serum; brilliant green agar (BGB132; tryptose agar base with 5% bovine serum, .001 μg/ml brilliant green, 25 U/ml Bacitracin, 100 μg/ml cycloheximide, 100 U/L Nystatin, 20 μg/ml vancomycin, 50 μg/ml trimethoprim, and 100 μg/ml EDTA), Kudzas Morse agar (KM; tryptose agar base with 5% bovine serum, 25 U/ml bacitracin, 6 U/ml polymyxin B, 100 μg/ml cycloheximide, and 1.4 μg/ml ethyl violet), and RBM agar, a selective medium for SRB51 (Hornsby *et al.*, 2000). Inoculated plates were incubated at 37°C in 5% CO₂ for 7 days.

**Culture Identification**

*Brucella* suspect cultures were identified on the basis of colony morphology, growth characteristics (Alton *et al.*, 1988), and growth on selective media. Isolates were identified as *Brucella spp.* by a polymerase chain reaction technique (PCR) using *Brucella*-specific primers to the omp2A region of the *Brucella* genome. Reactions consisted of 50 μl and contained 5 μl of suspect culture in tris-EDTA and 45 μl of reaction mixture consisting of 200 μM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim, Indianapolis, IN), 1 X PCR Buffer II (Perkin Elmer, Branchburg, NJ), 1.5 mM MgCl₂, 1.25 U AmpliTaq Gold polymerase (Perkin Elmer, Branchburg, NJ), and 0.2 mM of each upstream (5’-GCAACGTTGTCTCCACTC-3’) and downstream (5’-GTATCAGGCTACGCAGAAGG-3’) primers selected from the omp2A sequences of *Brucella abortus* (Ficht *et al.*, 1989). The primers had 100% conservancy within the genomes of *Brucella suis* and *Brucella melitensis* according to BLAST analysis. Tris-EDTA and a culture of *Yersinia enterocolitica* O:9 served as negative controls, and *Brucella suis* strain 3B, a biovar 1 laboratory challenge strain, served as a positive control. Following a 10 minute activation at 95°C reaction preparations were cycled in a thermocycler (MJ Research Inc., Watertown, MA) for 40 cycles consisting of
30 sec. at 95°C, 30 sec. at 44°C, and 60 sec. at 72°C. Products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Animals were considered culture positive if at least one sample yielded a positive *Brucella* culture.

Cultures which were positive by the omp2A PCR assay were run in a second PCR assay in order to determine if they were SRB51. This PCR assay utilized SRB51 specific primers targeted toward the insertion sequence 711 (IS711) (Vemulpalli *et al.*, 1999; Bricker and Halling, 1995). Each reaction mixture consisted of a volume of 25 μl containing 2.5 μl of suspect culture in Tris-EDTA, 1X PCR reaction buffer containing 50 mM Tris, 1.5 mM MgCl₂, 10 mM KCl, 50 mM (NH₄)₂SO₄, pH=8.3 (Roche Molecular Biochemicals, Indianapolis, IN), 200 μM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim, Indianapolis, IN); 1X GC rich solution (Roche Molecular Biochemicals, Indianapolis, IN); 1.0 U of FastStart DNA polymerase (Roche Diagnostics, Basel, Switzerland); and 0.2 μM of each upstream (5’-TGCCGATCCTAAGGCGCTTCATTGCCAG-3’) and downstream (5’-GCCAACCAACCCAAATGCTCACAA-3’) primer. Thermocycling consisted of a single 5 min. incubation at 95°C followed by 40 cycles consisting of 15 sec. at 95°C, 30 sec. at 52°C, and 90 sec. at 72°C. Tris-EDTA and *B. suis* strain 3B served as negative controls, and SRB51 served as the positive control. Products were analyzed by electrophoresis on 2.0% agarose gels stained with ethidium bromide.

**Statistical Analysis**

Seroconversion and *Brucella* culture positivity rates between vaccinates which were initially seronegative and nonvaccinates which were initially seronegative were used to evaluate efficacy of RB51. Chi-square analysis was used to determine significance with significance defined as P<0.05 (PROC FREQ CHISQ, SAS Statistical Software, SAS Institute, Inc., Cary, NC).

Serologic responses to SRB51 were evaluated on a fold-difference basis. The quotients of the necropsy SRB51 dot blot titers divided by the prevaccination titers were converted to logarithmic values and compared between vaccinates and controls using Student’s t-test (PROC MEANS T, SAS Statistical Software, SAS Institute, Inc., Cary, NC).
The immugenicity of SRB51 was also evaluated using Chi-square analysis. Positive dot blot titer rates were defined as a four-fold or greater increase in titer between day 0 and necropsy samples. Vaccinates were compared to nonvaccinates.

**Results**

**Serology**

The seroprevalence rate for *Brucella* of all initially captured animals was 34.5% (84/244). The overall seroprevalence rate of recaptured animals was 42.5% (34/80). Data related to the efficacy of SRB51 are summarized in Table 1. The seroconversion rates among vaccinates which were initially seronegative was 32.0% (9/28). The seroconversion rate among nonvaccinated controls which were initially seronegative was 40% (10/25).

Data related to SRB51 dot blot titers are summarized in figures 2 and 3. The mean SRB51 titers among vaccinates were 1:226 prevaccination and 1:425 at the time of necropsy and 1:250 among controls upon initial capture and 1:288 at necropsy (Figure 1). This represented a mean of 3.28-fold increase for vaccinates and a mean 2.60-fold increase for controls (p=0.45) (Figure 2). Mean SRB51 titers for animals which were seronegative for *Brucella* at the time of initial capture were 1:205 prevaccination and 1:465 at the time of necropsy for vaccinates and 1:267 upon initial capture and 1:503 at the time of necropsy for controls. This results in a mean of 5.01-fold increase for vaccinates and 3.90-fold increase for controls (p=0.50).

**Culture**

Overall *Brucella* culture positivity rate was 77.5% (62/80). Six animals were culture positive for SRB51. Five of these animals were positive for both SRB51 and *Brucella spp.* A single animal was positive for only SRB51. Forty-four of the recaptured animals were vaccinates; therefore, SRB51 has a persistence rate of 13.6%.

In animals which were initially seronegative, *Brucella* field strains were recovered from 64.0% (16/25) of nonvaccinated and 67.9% (19/28) SRB51 vaccinates (Table 1).
Discussion and Conclusions

This is the first reported study of a field trial of SRB51 in a feral swine herd with enzootic brucellosis. The results of this study indicate that SRB51 had no effect in preventing seroconversion and infection with *Brucella spp* in seronegative feral swine when vaccinated with a standard vaccine dosage and returned to their natural environment.

Animals in this study were categorized into 4 groups: vaccinates which were initially seronegative, controls which were initially seronegative, vaccinates which were initially seropositive, and controls which were initially seropositive. Conclusions on the efficacy of SRB51 were made by comparing the populations of vaccinates and controls which were seronegative upon initial capture. There were no statistical differences between these two groups with respect to seroconversion (p=0.48) and *Brucella* culture positivity rate (P=0.45). Because SRB51 is a rough mutant which does not express the lipopolysaccharide O-side chain, the use of SRB51 in this feral swine herd had no effect on seroconversion to *Brucella spp*. as the 3 serologic assay used in this study utilize the lipopolysaccharide O-side chain of *Brucella abortus* as the antigen.

Vaccinated animals also failed to develop significant humoral immune response against SRB51. While many vaccinated animals did have four fold or greater increases in SRB51 titers, there was no significance in the number of animals which seroconverted when compared to controls (P=0.12). There were also no significant differences in necropsy sample SRB51 titer values between vaccinates and controls (P>0.05). There was a statistically significant difference in the factor increase of SRB51 titers between vaccinates which were initially seronegative and vaccinates which were initially seropositive (P=0.02). This may suggest that serostatus has an effect on vaccine immunogenicity; however, this is discredited by the lack of statistically significant differences in both absolute titer values and factor increase values between controls and vaccinates which were initially seronegative.

The results of this study are contrary to those of Lord and colleagues (1998). In that study SRB51 was used to parenterally vaccinate domestic gilts IM and orally at dosages of $10^6$-$10^9$ CFU. That study also used purified lipopolysaccharide O-side chain
from *B. abortus* or *B. suis* to vaccinate animals IM or PO. It was concluded that all vaccine preparations provided 100% protection based on lack of seroconversion, lack of abortions, and increased litter size over those of the control groups. That study was similar to the feral swine study of this paper in that the challenge dosage was not defined; vaccinated gilts were bred to boars which were previously determined to be infected and shedding *Brucella suis* in their semen. The study by Lord et al. (1998) also did not examine the *Brucella*-culture status of all animals in the study. Cultures were only performed on vaginal swabs from sows which aborted and on tissues from aborted fetuses. Whereas, samples from animals which had full-term pregnancies were not cultured.

Edmonds and colleagues (2001) also examined SRB51 vaccination of domestic swine with $10^9$-$10^{12}$ CFU/dose. That study concluded that some animals could develop a humoral immune response against SRB51 after SQ or oral vaccination. Unlike the feral swine study of this paper, the study by Edmonds did not have any nonvaccinated control animals to which the results of the SRB51 vaccinated animals could be statistically compared.

An interesting result from this study was the persistence of SRB51 in 6 vaccinated animals for as long as 14 months post vaccination. *B. abortus* usually produces a short-lived infection of only the draining lymph node of the area of infection in swine (Deyoe 1972). From all 6 of the SRB51 culture positive animals, SRB51 was isolated from tissues other than lymph nodes (prescapular, sternal, and medial retropharyngeal lymph nodes) draining the site of vaccination. This suggests that subsequent to vaccination, bacteremia and peripheral infection also occurred. This atypical response might be due to an immune-mediated enhancement of infection. Previous infection with *Brucella suis* in these animals might have allowed an increased incidence of infection and enhanced persistence of the antigenically related SRB51 by similar mechanisms as previously described for immune-mediated enhancement of infection of antigenically similar organisms (Mahalingam and Lidbury, 2003; Sullivan, 2001). The animals in this study were also heavily parasitized. There was evidence of nematode infestation, notably from *Stephanurus dentatus*, in almost every necropsied animal. Recently, it has been shown
that persistent helminths can down regulate TH1 cell mediated immune responses (Brady, et al., 1999) and allow enhancement of infection and persistence of bacteria (O’Neill et al., 2001). Perhaps, a similar process may be at least partially responsible for the unusual persistence of the SRB51 and for the markedly high Brucella culture positivity rate within this feral swine population.

The results of this study serve as an example for the lack of sensitivity of serological assays for diagnosing brucellosis in feral swine. When necropsy culture results were compared to necropsy serology results, the sensitivity of a combination of the three serological assays which were used was 54.1%. Previously published sensitivity rates for these assays in domestic swine were 20-67% for the card test, 83-100% for the standard tube test, and 80-94% for the FPA test (Paolo et al., 2000; Lord et al., 1997; Ferris et al., 1995). Anitgens used in these assays are from Brucella abortus which may at least partially account for the lack of sensitivity when evaluating swine presumably infected with Brucella suis.

The results of this study do not support the use of SRB51 as a suitable vaccine candidate to be used in the control of brucellosis in feral swine. This study also reiterates the lack of sensitivity of conventional serologic assays for diagnosing brucellosis in swine. Control of brucellosis in feral swine will likely require an efficacious vaccine which can be administered orally, and management of brucellosis in feral swine could be aided by the development of more sensitive serologic assays.

**Acknowledgements**

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**References**


Table 1. Efficacy of SRB51 in Feral Swine. Comparison between vaccinates and controls. All animals were seronegative at the time of primary capture.

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<th>Controls (n=25)</th>
<th>Vaccinates (n=28)</th>
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<tr>
<td>Seroconversion(^{ac})</td>
<td>40% (10/25)</td>
<td>32% (9/28)</td>
</tr>
<tr>
<td>Brucella culture +(^{bd})</td>
<td>64.0% (16/25)</td>
<td>67.9% (19/28)</td>
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\(^{a}\)Seroconversion defined as positive result on at least one of the following assays: Card Test, Standard Tube Test, and Fluorescence Polarization assay.

\(^{b}\)Brucella Culture + defined as culturing Brucella spp. one or more samples.

\(^{c}\)P=0.28

\(^{d}\)P=0.45
Figure 1. Serologic responses of feral swine to $\gamma$-irradiated SRB51 in a dot blot assay. Feral swine were vaccinated with either $1 \times 10^{10}$ CFU SRB51 vaccine or saline. Responses are presented as mean titer $\pm$SEM. Means with the same lowercase letter are significantly different ($P<0.05$).
Figure 2. Serologic responses of feral swine to γ-irradiated SRB51 in a dot blot assay expressed as the factor of increase between initial capture and necropsy sera. Feral swine were vaccinated with either 1 X10^{10} CFU SRB51 vaccine or saline at the time of initial capture. Responses are presented as mean quotient ±SEM.

\[ a \] P<0.05
CHAPTER 5. PARENTERAL VACCINATION OF DOMESTIC PIGS WITH *BRUCELLA ABORTUS* STRAIN RB51

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Abstract

**Objective**—To determine the immunogenicity and efficacy of *Brucella abortus* strain RB51 (SRB51) as a vaccine in domestic pigs.

**Animals**—68 six-week-old crossbred domestic pigs and 24 four-month-old gilts.

**Procedures**—In experiment 1, pigs were vaccinated IM (n = 51) with $2 \times 10^{10}$ CFUs of SRB51 or sham inoculated (17). Periodic blood samples were obtained to perform blood cultures, serologic evaluations, and cell-mediated immunity assays. Necropsies were performed at selected times between weeks 1 and 23 after vaccination to determine vaccine clearance. In experiment 2, gilts were similarly vaccinated (n = 18) or sham inoculated (8) and similar samples obtained after vaccination. Gilts were bred and challenged conjunctivally with $5.0 \times 10^7$ CFUs of virulent *Brucella suis* strain 3B. Necropsies were performed on gilts and on fetuses or neonates after abortion or parturition, respectively. Bacterial cultures and serologic evaluations were performed on samples obtained at necropsy to determine vaccine efficacy.

**Results**—Humoral and cell-mediated immune responses did not differ between vaccinates and controls. After vaccination, SRB51 was not isolated from blood cultures of either group and was isolated from lymphoid tissues of 3 pigs at 2 weeks (n = 2) and 4 weeks (1) after vaccination. No differences were found in isolation of *B suis* or in seroconversion between vaccinated and control gilts and between their neonates or aborted fetuses.
**Conclusions and Clinical Relevance**—Parenteral vaccination with SRB51 does not induce humoral or cell-mediated immune responses. Vaccination with SRB51 did not protect gilts or their neonates and fetuses from virulent challenge with *B suis*.

**Introduction**

Swine brucellosis, which is caused by the bacterium *Brucella suis*, is found in domestic or feral pigs on all inhabited continents.\(^1\) Manifestations of swine brucellosis range from subclinical infection to abortions and infertility.\(^1\) In addition to its effects on female reproduction cycles and pregnancy, swine brucellosis may also manifest as infection and inflammation of primary and secondary reproductive organs of males as well as multicentric arthritis, diskospondylitis, and lymphadenitis in all aged pigs of both sexes.\(^1\) Of all the *Brucella* spp, *B suis* is most noteworthy for causing chronic and persistent infections.\(^2,3\) *Brucella suis* is a zoonotic agent that has been known to infect slaughter house workers, farm workers, and hunters who have been exposed to tissues from *B suis* infected pigs.\(^4,5\)

In the United States the Cooperative State-Federal Brucellosis Eradication Program has led to the near elimination of swine brucellosis within domestic pigs. Currently, 49 states and Puerto Rico are classified as free of swine brucellosis (ie, stage III) while Texas is classified as stage II (more than 1 *B. suis* infected herd identified in the past 2 years).\(^6\) However, periodic, isolated outbreaks of swine brucellosis still occur within the United States. Most of these outbreaks are attributed to contact with feral pigs. Feral pigs have also been responsible for infecting cattle with *B suis*.\(^7\)

The reduction and near eradication of swine brucellosis from domestic herds within the United States has been achieved through a systematic program of herd testing, slaughter testing, trace-back of reactors, and disposal of reactor herds. With the near complete eradication of swine brucellosis from domestic pigs in the United States, a reemergence of interest exists in new strategies, including candidate vaccines, to control swine brucellosis particularly in light of the wide distribution of *Brucella*-infected feral pigs across the United States.
**Brucella abortus** strain RB51 is a laboratory derived lipopolysaccharide O-side chain-deficient mutant of *B abortus* strain 2308.\(^8\) *Brucella abortus* strain RB51 induces protective immunologic responses in cattle and bison against challenge-exposure with virulent *B abortus* strains.\(^9,10,11\) *Brucella abortus* strain RB51 also does not induce antibody responses that react with conventional brucellosis serologic surveillance tests.\(^12,13\) Initial reports suggested that SRB51 induces an immune response and prevents transmission of *B suis* in pigs.\(^14,15\) The purposes of the study reported here were to determine the potential of parenteral administration of SRB51 to elicit an immune response as well as protection from challenge with virulent *B suis* in domestic pigs.

**Materials and Methods**

**Bacterial cultures**

A master seed stock of SRB51 was obtained.\(^4\) After 1 passage on TSA, the seed stock was designated ARS/1. For experimental use in serologic and lymphocyte proliferation assays, SRB51 (ARS/1) bacteria were grown on TSA\(^b\) for 48 hours at 37°C. Resulting cultures were suspended in PBS (0.15M NaCl) solution at a concentration of 1.3 × 10\(^{12}\) CFUs/mL and inactivated by γ-irradiation (1.4 × 10\(^6\) rad). After irradiation, suspensions were washed in 0.15M NaCl solution and stored in 1.0 mL aliquots at −70°C.

For vaccination of pigs, SRB51 (ARS/1) was expanded on TSA for 48 hours at 37°C with 5% CO\(_2\). Harvested bacteria were suspended in PBS (0.15M NaCl) solution and then diluted to a concentration of 1.0 × 10\(^{10}\) CFUs/mL in PBS (0.15M NaCl) solution by use of an optical density method and spectrophotometer\(^c\). The final concentration was determined by standard plate counts on TSA after a 5-day incubation period at 37°C and 5% CO\(_2\).

The challenge culture *Brucella suis* strain 3B (biovar 1) was originally obtained from an aborted fetus of a sow exposed to a polyvalent suspension of 3 strains of *B suis*. The 3 strains had previously been isolated from boars originating from 3 sources. *Brucella suis* strain 3B has been maintained as a lyophilized culture since 1942. *Brucella suis* strain 3B was grown on TSA for 48 hours at 37°C with 5% CO\(_2\). Bacteria were
harvested and suspended in PBS (0.15M NaCl) solution and diluted to a concentration of 1 × 10^9 CFUs/mL by use of an optical density method and spectrophotometer. The final concentration was determined by standard plate counts on TSA after a 5-day incubation period at 37°C and 5% CO₂.

**Swine experiments and study design**

The work reported herein was performed under the approval of the Institutional Animal Care and Use Committee of the National Animal Disease Center (Ames, IA). Vaccine clearance and induction of an immune response were initially assessed in weaned pigs (experiment 1). A total of sixty-eight 6-week-old, crossbred domestic pigs were inoculated IM in the right cervical area with either 2.0 mL of PBS (0.15M NaCl) solution alone (n = 17) or 2.0 mL of PBS (0.15M NaCl) solution containing 2.0 × 10^10 CFUs of SRB51 (51). All pigs were commingled in a single pen and fed ad libitum.

Induction of an immune response and protection from virulent challenge with *B. suis* strain 3B were assessed in crossbred domestic gilts (experiment 2). A total of twenty-four 4-month-old gilts were inoculated IM in the right cervical area with 2.0 mL of PBS (0.15M NaCl) solution containing 2 × 10^10 CFUs of SRB51 (n = 18) or sham inoculated with 2.0 mL of PBS (0.15M NaCl) solution (8). At approximately 10 months of age, gilts were bred by artificial insemination. Pregnancy was verified by use of ultrasonography. Fourteen vaccinates and 8 control gilts were successfully bred and challenged at approximately day 75 of gestation by bilateral conjunctival administration of 5.0 × 10^7 CFUs of *B. suis* strain 3B (volume, 50 μL/eye).

**Serologic evaluation**

Blood was collected for serologic evaluation by cranial vena cava puncture in experiment 1 (weaned pig group) at weeks 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 after vaccination and in experiment 2 (gilt group) at weeks 0, 2, 4, 8, 12, 16, 20, and 24 after vaccination. Blood samples were allowed to clot and serum was separated by centrifugation. Serum was divided into 1 mL aliquots, and stored at −70°C until assays were performed. Serum antibody titers to SRB51 were determined by use of a previously described dot blot assay in which γ-irradiated SRB51 was used as the antigen and
peroxidase-labeled rabbit anti-swine IgG was used as the secondary antibody at a dilution of 1:500. Seroconversion as a result of challenge with the *B suis* strain was determined by use of fluorescence polarization, standard tube agglutination, and card agglutination assays with previously described methods that use *B abortus* antigen. Blood was collected on day 0 of challenge from all gilts and at necropsy from all gilts, neonates, and fetuses. Pigs that had a positive reaction on ≥1 of the 3 serologic assays were considered seroreactors.

**PBMC proliferation assays**

Blood samples (45 mL) were collected into acid-citrate dextrose solution from the cranial vena cava at weeks 8, 12, 14, 16, 18, and 20 after vaccination from pigs in experiment 1 and at weeks 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 after vaccination from the gilts in experiment 2. Peripheral blood mononuclear cells were enriched by density centrifugation with a Ficoll-sodium diatrizoate gradient.

Fifty microliters of each cell suspension containing $5 \times 10^5$ PBMCs were added to each of 2 separate flat-bottom wells of 96-well microtiter plates that contained 100 μL of RPMI 1640 medium only or RPMI 1640 medium containing γ-irradiated SRB51 (10$^5$ to 10$^9$ bacteria/well). Cell cultures were incubated for 4 days at 37°C under 5% CO$_2$. Plates were then pulsed with 1.0 μCi of [$^3$H] thymidine/well for 18 hours. Cells were harvested onto glass filter mats and counted for radioactivity in a liquid scintillation counter.

**Necropsy examination**

All pigs were euthanatized by administration of sodium pentobarbital into the cranial vena cava. Tissues collected for bacterial culture were collected with an aseptic technique, placed into individual containers, and immediately frozen at −70°C until processed. Tissues collected for histologic evaluation were immediately placed in neutral-buffered 10% formalin, processed by routine paraffin embedding techniques, cut in 4-μm-thick sections, and stained with H&E.

In experiment 1, 4 vaccinates and 1 control pig were necropsied at weeks 1, 2, 3, 4, 5, and 6 after vaccination. Five vaccinates and 2 controls were necropsied at weeks 8, 12, 16, and 20 after vaccination, and the remaining 7 vaccinates and 3 controls were
necropsied at week 23 after vaccination. Blood and urine were collected for bacterial culture at necropsy. The following tissues were collected for bacterial culture and histologic evaluation: liver, spleen, kidney, lung, pharyngeal tonsils, and lingual tonsil as well as prescapular, medial retropharyngeal, sternal, tracheobronchial, mediastinal, gastrohepatic, ileocecal, jejunal, renal, iliac,inguinal, prefemoral, popliteal, mandibular, and parotid lymph nodes.

In experiment 2, all neonates or fetuses were euthanatized and necropsied within a few hours of parturition or abortion, respectively. Samples of whole blood, CSF, and stomach contents, and rectal swab specimens were obtained for bacterial culture, and lung, liver, spleen, and kidney tissue specimens were obtained for bacterial culture and histologic evaluation. Gilts were euthanatized and necropsied within 2 days of parturition (or abortion). Samples of whole blood, milk, and urine, and vaginal swab specimens were obtained for bacterial culture, and lung, liver, spleen, kidney, mammary gland, uterus, and placenta tissue specimens as well as tracheobronchial, prescapular, medial retropharyngeal, mandibular, parotid, sternal, iliac, prefemoral, popliteal, gastrohepatic, and ileocecal lymph nodes were obtained for bacterial culture and histologic evaluation.

**Bacterial culture**

In experiment 1, whole blood was collected for bacterial culture at days 0, 7, 10, 14, 17, 21, 24, 28, and weeks 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, and 23 after vaccination. In experiment 2, whole blood was collected for bacterial culture at day 0 and weeks 2, 4, 8, 12, 16, 20, and 24 after vaccination. Whole blood samples and conjunctival, nasal, and vaginal swab specimens were collected after virulent challenge with *B. suis* strain 3B on days 0, 3, 7, and 10, and weekly thereafter until parturition or abortion.

Whole blood for bacterial culture was immediately placed into tryptose broth containing acid citrate dextrose (1:1, vol/vol) and incubated for 7 days at 37°C and 5% CO₂ prior to plating on *Brucella* selective media. Urine, stomach contents, CSF, and milk were incubated in tryptose broth containing 5% bovine serum (1:3, vol/vol) for 7 days at 37°C and 5% CO₂ prior to plating on *Brucella* selective media. After thawing, tissues were individually ground in approximately 10% (wt/vol) sterile PBS solution (pH 7.2)
with sterile glass grinders. Aliquots (100 μL) of each tissue homogenate or preincubated tryptose broth (blood, urine, stomach contents, CSF, and milk) were plated on 4 Brucella selective media. Media included tryptose agar containing 5% bovine serum; brilliant green agar (containing tryptose agar base with 5% bovine serum, brilliant green [0.001 μg/mL], bacitracin [25 U/mL], cycloheximide [100 μg/mL], nystatin [100 U/L], vancomycin [20 μg/mL], vancomycin [50 μg/mL], and EDTA [100 μg/mL]); Kuzdas-Morse selective medium (containing tryptose agar base with 5% bovine serum, bacitracin [25 U/mL], polymyxin B [6 U/mL], cycloheximide [100 μg/mL], and ethyl violet [1.4 μg/mL]); and RBM agar, a selective medium for SRB51. Inoculated plates were incubated at 37°C in 5% CO2 for 7 days.

**Bacterial culture results**

Suspect cultures of *Brucella* spp were identified on the basis of colony morphology, growth characteristics, and growth on selective media. Isolates were identified as *Brucella* spp by use of a PCR technique with *Brucella*-specific primers to the omp2A region of the *Brucella* genome. Reactions consisted of 50 μL and contained 5 μL of the suspect culture of *Brucella* spp in tris-EDTA and 45 μL of reaction mixture consisting of 200μM each of dATP, dCTP, dGTP, and dTTP, 1× PCR buffer II, 1.5mM MgCl2, 1.25 units of Taq polymerase, and 0.2mM of each upstream (5’-GCAACGGTGTTCTTCCACTC-3’) and downstream (5’-GTATCAGGCTACGCAGAAGG-3’) primer selected from the omp2A sequences of *Brucella abortus*. Primers had 100% conservancy within the genomes of *Brucella suis* and *Brucella melitensis* according to the results of a basic local alignment search tool (ie, BLAST) analysis. Tris-EDTA and a culture of *Yersinia enterocolitica* O:9 served as negative controls, and *Brucella suis* strain 3B served as a positive control. Following a 10-minute activation at 95°C, reaction preparations were cycled in a thermocycler for 40 cycles consisting of 30 seconds at 95°C, 30 seconds at 44°C, and 60 seconds at 72°C. Products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Pigs were considered infected with *B suis* strain 3B if ≥ 1 sample yielded a positive *Brucella* culture result.
Cultures that had positive results for Brucella spp by the omp2A PCR assay were run in a second PCR assay to determine whether they were SRB51. This PCR assay used SRB51 specific primers targeted toward the insertion sequence 711. Each reaction mixture consisted of a volume of 25 μL containing 2.5 μL of the suspect culture of Brucella spp in Tris-EDTA, 1× PCR reaction buffer containing 50mM Tris, 1.5mM MgCl₂, 10mM KCl, 50mM (NH₄)₂SO₄, pH 8.3, and 200μM each of dATP, dCTP, dGTP, and dTTP; 1× guanine-cytosine rich solution; 1.0 unit of DNA polymerase; and 0.2μM of each upstream (5’-TGCCGATCATTAGGGCCTTCATTGCCAG-3’) and downstream (5’-GCCAACCAACCCAAATGCCTCACAA-3’) primer. Thermocycling consisted of a single 5 minute incubation at 95°C followed by 40 cycles consisting of 15 seconds at 95°C, 30 seconds at 52°C, and 90 seconds at 72°C. Tris-EDTA and B suis strain 3B served as negative controls, and SRB51 served as the positive control. Products were analyzed by electrophoresis on 2.0% agarose gels stained with ethidium bromide.

**Statistical analysis**

For all analyses a level of \( P < 0.05 \) was used to determine significant differences between vaccinates and controls. Serologic response data to SRB51 were converted to the logarithm of the titer for analysis. Resulting values were compared between vaccinates and controls by use of a repeated measures, general linear model procedure. Proliferative responses to SRB51 were converted to the logarithm of the mean cpm and compared between vaccinates and controls by use of a general linear model procedure and least squared means. A \( \chi^2 \) test was used to determine differences in positive bacterial culture results and seropositivity between neonates and fetuses from vaccinated versus control gilts. The Fisher exact test was used to determine differences between vaccinates and controls on a per litter basis and to determine differences in positive bacterial culture results, seroconversion, and histologic lesions between vaccinated and control gilts or between neonates and fetuses from vaccinated or control gilts.
Results

Serologic evaluation

The SRB51 dot blot titers were analyzed (Figure 1 and 2) to determine serum antibody titers to SRB51. No significant differences in serum antibody titers to SRB51 were found between vaccinates and controls in both experiments with the exception of week 22 in experiment 1; however, at this time point the mean titer of the control group was significantly higher than the vaccinated group.

Seroconversion rates for control and vaccinated gilts that were challenged with \textit{B suis} strain 3B during gestation were 7 of 8 and 11 of 14 gilts, respectively, \((P = 0.40)\). Seropositivity rate was 27.8\% (25/90) for neonates and fetuses from control gilts and 35.1\% (60/171) for neonates and fetuses from vaccinated gilts \((P = 0.23)\).

PBMC proliferation assays

Analysis of data from PBMC proliferation assays revealed that at all time points in both experiments no significant difference was found in mean cpm between the vaccinated and control groups (Figures 3 and 4). At each time point the pokeweed mitogen positive control wells yielded mean cpm of \(> 100,000\) for the control and vaccinated groups.

Bacterial culture results

In experiment 1, SRB51 was isolated from 3 of 51 vaccinated pigs at the following time points: the lingual tonsil and sternal lymph node in 1 pig at 2 weeks after vaccination; the tracheobronchial lymph node from 1 pig at 2 weeks after vaccination; and the prescapular lymph node from 1 pig at 4 weeks after vaccination. \textit{Brucella abortus} strain RB51 was not isolated from blood cultures from any pigs at any time point in experiments 1 and 2, and SRB51 was also not isolated from samples obtained at necropsy from sham inoculated control pigs in experiment 1.

Recovery of \textit{B suis} after inoculation and from tissues collected at necropsy from control and vaccinated gilts and their neonates and fetuses were recorded (Table 1). Polymerase chain reaction analysis of all isolates obtained after inoculation also at necropsy confirmed that none were SRB51. Pigs were considered infected with \textit{B suis}
strain 3B if \( \geq 1 \) sample (ie, tissues, blood, swab specimens, fluids) yielded a positive *Brucella* culture result. No significant differences in positive bacterial culture result rates were found between control and vaccinated gilts for samples from the after inoculation-antemortem period \( (P = 0.14) \) and from samples obtained at necropsy \( (P = 0.39) \). Likewise, no significant differences were found in positive bacterial culture result rates between neonates and fetuses from control gilts or vaccinated gilts when examined on a per neonate-fetus basis \( (P = 0.46) \) or on a per litter basis \( (P = 0.47) \).

**Lesions**

No gross lesions were observed in pigs of experiment 1, and with the exception of purulent endometrial exudate in 1 vaccinated gilt, no gross lesions were observed in gilts of experiment 2. No noteworthy histologic lesions were found in pigs from experiment 1. Histologic lesions in the gilts from experiment 2 and their neonates and fetuses were recorded. Purulent lymphadenitis characterized by mild to moderate numbers of neutrophils present within sinusoids of interfollicular areas of multiple lymph nodes was present in 5 pigs (5 vaccinates). Lymph nodes affected included medial retropharyngeal (2), mandibular (1), gastrohepatic (1), sternal (1), popliteal (1), iliac (1), and tracheobronchial (1). Portal hepatitis characterized by low to moderate number of neutrophils, lymphocytes, and plasma cells surrounding portal vessels and bile ducts was present in 5 pigs (2 controls and 3 vaccinates). Five gilts (2 controls and 3 vaccinates) had purulent endometritis characterized by low to moderate numbers of neutrophils within the mucosa and lamina propria of the uterus. In 1 control pig moderate numbers of lymphocytes and plasma cells were present in addition to neutrophils. Five pigs (1 control and 4 vaccinates) had interstitial nephritis characterized by multifocal areas of low to moderate number of neutrophils, lymphocytes, and plasma cells within cortical interstitial areas. No significant \( (P = 0.34) \) difference was found between the number of control and vaccinated pigs that had histologic lesions consistent with *Brucella* infection.

Histologic lesions consistent with *Brucella* infection were present in 21.2% (19/90) of neonates and fetuses from control gilts and 23.3% (40/171) of neonates and fetuses from vaccinated gilts. Diffuse, mild to moderate, purulent interstitial pneumonia characterized by low to moderate numbers of neutrophils infiltrating alveolar septa was
present in 18.9% (17/90) of neonates and fetuses from control gilts and 19.9% (34/171) of neonates and fetuses from vaccinated gilts. Multifocal, purulent, portal hepatitis characterized by mild to moderate numbers of neutrophils surrounding vessels and bile ducts within portal areas of the liver was present in 2 neonates and fetuses from control gilts and 6 neonates and fetuses from vaccinates gilts. No significant ($P = 0.83$) difference was found between the number of neonates and fetuses from control gilts and vaccinated gilts that had histologic lesions consistent with *Brucella* infection.

**Discussion**

Results of our study indicate that single dose parenteral vaccination with SRB51 is not sufficiently immunogenic to protect against virulent challenge with *B suis* strain 3B in domestic pigs. In experiments 1 and 2, vaccinated pigs failed to develop antibody titers against $\gamma$-irradiated SRB51 that were significantly higher than those of the nonvaccinated controls. Serum antibody titers examined in cattle in which SRB51 has been found to be immunogenic and efficacious were significantly higher in vaccinated pigs than in nonvaccinated controls at weeks 4 to 20 after vaccination.\textsuperscript{23, 24} Also, no significant difference was found in seroconversion between control and vaccinated gilts and between neonates and fetuses from control and vaccinated gilts after challenge with virulent *B suis*.

No evidence exists from our study indicating that SRB51 elicits a strong cell-mediated immune response after parenteral vaccination in pigs. Results of PBMC proliferation assays after stimulation with $\gamma$-irradiated SRB51 did not significantly differ between controls and vaccinated pigs in experiments 1 and 2. *Brucella abortus* strain RB51 has been shown to elicit robust proliferative responses in other species. Bison parenterally vaccinated with 1.2 to $6.1 \times 10^{10}$ CFUs of SRB51 have significantly higher PBMC proliferation responses than nonvaccinated controls at weeks 12 and 18 after vaccination,\textsuperscript{25} and the same dosage of SRB51 in a subsequent study\textsuperscript{11} was demonstrated to be protective in bison. Likewise, cattle parenterally vaccinated with $1 \times 10^{10}$ CFUs of SRB51 had PBMC proliferative responses that were significantly higher than those of controls at weeks 10, 12, 14, and 16 after vaccination, and cattle parenterally vaccinated
with $3 \times 10^9$ CFUs of SRB51 had PBMC proliferative responses that were significantly higher than those of controls at weeks 14 and 18 after vaccination.\textsuperscript{23,24} Both doses were associated with protection from virulent \textit{B abortus} challenge.\textsuperscript{23,24} It has been shown that pigs that are infected with \textit{B suis} are capable of eliciting a significantly higher PBMC proliferative response over noninfected controls.\textsuperscript{26}

Data from the weaned pigs of experiment 1 suggest that SRB51 is cleared quickly in pigs. Isolation of SRB51 was achieved in only 3 pigs (2 at week 2 after vaccination and 1 at week 4 after vaccination). The elicitation of a protective immune response has been associated with longer persistence of SRB51 within regional lymph nodes. In bison bacterial titers of SRB51 were found to peak at week 2 after vaccination and steadily decline through week 18 after vaccination with total vaccine clearance achieved by week 24 after vaccination.\textsuperscript{25,27,28} In cattle, SRB51 has been shown to persist until week 14 after vaccination within the regional lymph nodes of some cattle that were vaccinated with protective doses.\textsuperscript{29}

The hallmark of \textit{Brucella} vaccine efficacy has long been the reduction in recovery of \textit{Brucella} organisms in maternal and fetal tissues after virulent challenge.\textsuperscript{11,24,29} Our study failed to show a significant reduction in the recovery of \textit{B suis} organisms on a per gilt, per neonate-fetus, or per litter basis.

Results of our study are contrary to those reported by Lord et al.\textsuperscript{15} In their study SRB51 was used to parenterally vaccinate domestic gilts IM and also PO at doses of $10^6$ to $10^9$ CFUs. In that study the lipopolysaccharide O-side chain from \textit{B abortus} or \textit{B suis} was also used to vaccinate pigs IM or PO. It was concluded that all vaccine preparations provided 100% protection on the basis of the lack of seroconversion, lack of abortions, and increased litter size over those of the control groups. A major difference between the study by Lord et al\textsuperscript{15} and our study is that in their study the challenge dose was not defined; vaccinated gilts were bred to boars previously determined to be shedding \textit{B suis} in their semen; however, the culture status of the semen from the boars used for sire was not determined on the days of insemination. Our study used a defined challenge dose of a single strain of virulent \textit{B suis} for conjunctival administration. In the study by Lord et al\textsuperscript{15} the \textit{Brucella}-culture status of all pigs was not examined.\textsuperscript{15} Cultures were only performed
on vaginal swab specimens from sows that aborted and on tissues from aborted fetuses. Samples from pigs that had full-term pregnancies were not cultured. In our study we determined the culture status on the basis of a full complement of tissues from vaccinated and control gilts as well as from their neonates and fetuses to assess the efficacy of vaccination with SRB51.

Edmonds et al\textsuperscript{14} also examined SRB51 vaccination of domestic pigs with $10^9$ to $10^{12}$ CFUs/dose.\textsuperscript{14} Results of that study indicate that some pigs can develop a humoral immune response against SRB51 after SC or oral vaccination. Unlike our study, the study by Edmonds et al\textsuperscript{14} did not have any nonvaccinated control pigs to which the results of the SRB51 vaccinated pigs could be compared.

Results of our study are similar to those of other studies\textsuperscript{30,31} in which \textit{B. abortus} strain 19 was examined as a vaccine candidate for control of swine brucellosis. \textit{Brucella abortus} strain 19 was found to not confer demonstrable immunity and protection against virulent challenge with \textit{B. suis}.\textsuperscript{30,31} Cedro et al,\textsuperscript{32,33} however, reported efficacy of a live \textit{B. abortus} vaccine strain when coadminister with heat-killed \textit{B. suis} and lipopolysaccharide from \textit{B. suis}.\textsuperscript{32,33} This compound vaccine was found to decrease abortion rates as well as culture recovery of \textit{B. suis}.

Although \textit{Brucella} vaccines traditionally have not been 100\% efficacious under experimental conditions in preventing maternal infection, fetal infection, and lesions, the SRB51 and \textit{B. abortus} strain 19 vaccines greatly reduce the occurrence of all of these after virulent challenge in cattle. In our study none of these conditions were met; therefore, SRB51 does not appear to be a suitable vaccine candidate for the control of \textit{B. suis} infection in pigs.

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\textsuperscript{f}Boehringer Mannheim, Indianapolis, IN, USA
8. AmpliTaq Gold polymerase, Perkin Elmer, Branchburg, NJ, USA
9. MJ Research Inc., Watertown, MA, USA
10. FastStart DNA polymerase, Roche Molecular Biochemicals, Indianapolis, IN, USA
11. Roche Diagnostics, Basel, Switzerland
12. PROC GLM LSMEANS, SAS Statistical Software, SAS Institute, Inc., Cary, NC, USA
13. PROC FREQ CHISQ, SAS Statistical Software, SAS Institute, Inc., Cary, NC, USA

References


Table 1—Recovery of *Brucella suis* from individual neonates and farrowed litters at necropsy and gilts after inoculation and at necropsy.*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neonates and fetuses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. from vaccinates</td>
<td>96 (56.1%)</td>
<td>75 (43.9%)</td>
</tr>
<tr>
<td>No. from control gilts</td>
<td>55 (61.1%)</td>
<td>35 (38.9%)</td>
</tr>
<tr>
<td><strong>Litters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. from vaccinates</td>
<td>12 (85.7%)</td>
<td>2 (14.3%)</td>
</tr>
<tr>
<td>No. from control gilts</td>
<td>7 (87.5%)</td>
<td>1 (12.5%)</td>
</tr>
<tr>
<td><strong>Gilts after inoculation, antemortem</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of vaccinates</td>
<td>8 (57.1%)</td>
<td>6 (42.9%)</td>
</tr>
<tr>
<td>No. of control gilts</td>
<td>7 (87.5%)</td>
<td>1 (12.5%)</td>
</tr>
<tr>
<td><strong>Gilts at necropsy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of vaccinates</td>
<td>12 (85.7%)</td>
<td>2 (14.3%)</td>
</tr>
<tr>
<td>No. of control gilts</td>
<td>8 (100%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*Gilts were challenged with $5.0 \times 10^7$ CFUs of *B suis* strain 3B on day 75 of gestation.
Figure 1—Mean ± SEM serum antibody titer to SRB51 in vaccinated or control weaned pigs on the basis of γ-irradiated SRB51 dot-blot assay results. Pigs were vaccinated with 2.0 × 10^{10} CFUs of SRB51 (closed bars; n = 51) or sham inoculated with an equal volume of PBS solution (open bars; n = 17).

![Graph](image1)

Figure 2—Mean ± SEM serum antibody titer to SRB51 in vaccinated or control gilts on the basis of γ-irradiated SRB51 dot-blot assay results. Gilts were vaccinated with 2.0 × 10^{10} CFUs of SRB51 (closed bars; n = 18) or sham inoculated with an equal volume of PBS solution (open bars; n = 8).

![Graph](image2)
Figure 3—Mean cpm ± SEM in PBMC blastogenesis assays of weaned pigs vaccinated with $2.0 \times 10^{10}$ CFUs of SRB51 (closed bars [stimulated], gray bar [not stimulated]; n = 9) or sham inoculated with an equal volume of PBS solution (open bars [stimulated], hatched bar [not stimulated]; n = 6).

Figure 4—Mean cpm ± SEM in PBMC blastogenesis assays of gilts vaccinated with $2.0 \times 10^{10}$ CFUs of SRB51 (closed bars [stimulated], gray bar [not stimulated]; n = 18) or sham inoculated with an equal volume of PBS solution (open bars [stimulated], hatched bar [not stimulated]; n = 8).
CHAPTER 6. RESULTS OF VACCINE TRIALS USING A NATURALLY ROUGH MUTANT OF BRUCELLA SUIS IN SWINE

A paper submitted to the American Journal of Veterinary Research

William C. Stoffregen, DVM and Steven C. Olsen, DVM, PhD

Abstract

Objective – To determine the safety, immunogenicity, clearance, and efficacy of *Brucella suis* strain 353-1, a naturally rough mutant, as a vaccine in domestic swine.

Animals – Experiment 1 contained 21 inoculated, domestic swine and 6 age-matched, controls. Experiment 2 contained 84 vaccinated, domestic swine, 9 age-matched controls, and 4 age-matched sentinels.

Procedures – In experiment 1, pigs were conjunctivally inoculated with $5 \times 10^7$ CFU of 353-1. Periodic bleedings were performed for blood culture, serology, and cell-mediated immunity assays. Necropsies were performed at selected time periods between weeks 1 and 18 after inoculation to determine pathogenicity, distribution, and clearance of 353-1. In experiment 2, pigs were vaccinated orally or parenterally with live 353-1 or parenterally with adjuvanted, heat-killed 353-1 and similar postvaccination samples taken. Vaccinated, control, and sentinel pigs were challenged conjunctivally with $5.0 \times 10^7$ CFU virulent *B. suis* strain 3B. Necropsies were performed, and bacterial culture was performed on necropsy samples to determine vaccine efficacy.

Results – *B. suis* 353-1 was nonpathogenic and cleared by week 20 in inoculated and vaccinated swine. Parenteral and oral vaccination induced significant cell-mediated immune responses, and parenteral vaccination elicited a significant humoral response. There appeared to be no shedding of 353-1 from parenterally vaccinated animals. Recovery of the virulent challenge strain *B. suis* 3B was significantly lower in some tissue from orally and parenterally vaccinated animals compared to controls.
Conclusions – *B. suis* 353-1 is a stable, rough mutant which when used as a parenteral or oral vaccine can induce a significant immune response and confer a partial level of protection from challenge with virulent *B. suis*.

**Introduction**

The *Brucellae* compose a group of genetically homogenous, facultative, intracellular pathogens which infect dozens of terrestrial and marine mammal species worldwide.\(^1,2\) *Brucellae* have been conventionally divided into 8 genera based on the primary animal species which they infect; however, due to the high degree of genetic similarity, it has been proposed that all *Brucellae* are members of a single genus (*B. melitensis*).\(^3,4\) The specie *Brucella suis* is divided into 5 biovars: biovars 1 and 3 infect primarily swine and are found in North America; biovar 2 is primarily a European biovar which infects swine and has also been found to be maintained in European hares; biovar 4 is enzootically found in many reindeer and caribou herds; and biovar 5 infects murine species.\(^5\)

Swine brucellosis was first described in the United States by Traum in 1914.\(^6\) The disease syndrome in swine is characterized by abortions, stillbirths, and infertility in sows.\(^5\) Chronically infected boars may also suffer from decreased fertility and libido due to infections associated with pyogranulomatous and fibrosing lesions of the testis, epididymis, seminal vesicle, prostate, and bulbourethral gland. Other lesions associated with *Brucella* infection in swine are pyogranulomatous lymphadenitis, splenitis, hepatitis, arthritis, and diskospondilitis.\(^5,7,8\)

Swine brucellosis still occurs enzootically in parts of South America, sub-Saharan Africa, and Asia, but it has essentially been eradicated from domestic swine in the US with only sporadic outbreaks which are associated with feral swine contact.\(^9,10,11,12\) Elimination of swine brucellosis from the US is the result of the Cooperative State-Federal Brucellosis Eradication Program which relied on the principles of serological herd testing and elimination of seroreactive herds. The efforts of this program were undoubtedly aided by the drastic changes in swine production and swine farm management and biosecurity. Brucellosis is enzootic in feral swine herds within the US.
Feral swine not only threaten the stability of the brucellosis eradication campaign but also serve as a reservoir of pseudorabies virus. Feral swine associated outbreaks of brucellosis have not only occurred in swine but also cattle and humans. With feral swine becoming an increasingly popular game animal its distribution and numbers have dramatically increased in recent decades. This has lead to an increased interest in dealing with brucellosis in feral swine in order to prevent further transmission to domestic livestock and humans.

There have been limited studies dealing with the prevention of swine brucellosis with vaccines. Early studies with the smooth strain *B. abortus* S19 showed a lack of immunologic response and protection. Initial studies with the rough vaccine strain *B. abortus* RB51 reported that this vaccine induced a humoral immune response in swine and protected vaccinated swine 100% from challenge with *B. suis*. However, more controlled studies failed to agree with these initial reports. The later studies showed that swine parenterally vaccinated with RB51 failed to develop a cell-mediated and humoral immune response, and these lack of responses were associated with a failure of protection in a pregnant gilt challenge model. Despite its failure to induce immune responses in swine, RB51 has been shown to induce significant immune responses in cattle and bison and protect these species from challenge with virulent *B. abortus*. RB51 induces these protective immune responses despite being deficient of the lipopolysaccharide O-side chain, a property which has the advantage of not inducing humoral immune responses which would make vaccinated animals reactors to standard *Brucella* serological assay.

Herein, studies dealing with a natural rough mutant of *B. suis* are reported. These experiments were performed to determine the potential of this new rough *B. suis* strain as a vaccine which might serve as a tool for the control of brucellosis in domestic and feral swine populations. The studies were designed to assess pathogenicity, distribution, and clearance as well as the ability of live oral and parenteral and adjuvanted, heat-killed preparations of this rough *B. suis* strain to induce significant immune responses and confer protection from challenge with virulent *B. suis*. The *in vitro* and *in vivo* stability of the rough phenotype were also assessed.
Materials and Methods

Bacterial Cultures and Vaccine Preparation

The natural rough mutant of *B. suis* used in the vaccine trials reported herein was isolated from the urine of a feral boar from Georgetown County, South Carolina and given the arbitrary designation *B. suis* 353-1 (353-1). A master seed stock of 353-1 was prepared by the propagation of a single colony from the original isolation plate on tryptose agar\(^a\) containing 5% bovine serum (TSA) and stored at -80° C until vaccine or reagent preparation. For experimental use in serology and lymphocyte proliferation assays and for killed vaccine preparation, 353-1 bacteria were grown on TSA for 48 hours at 37° C. The resulting cultures were suspended in 0.15 M phosphate buffered saline (PBS), and the bacterial concentration was determined by serial dilutions and plate counts on TSA. The culture was then inactivated by incubation at 60° C for 2 hours. Inactivation was confirmed by plating the culture suspension on TSA. After inactivation, culture suspensions were stored at -80° C.

For conjunctival inoculation and parenteral and oral vaccination of swine, 353-1 was expanded on TSA for 48 hours at 37° C with 5% CO\(_2\). The harvested bacteria were suspended in 0.15 M PBS and then diluted to a concentration of 1.0 X 10\(^9\) CFU/ml for conjunctival inoculation or 1.0 X 10\(^10\) CFU/ml in 0.15 M PBS using an optical density method and spectrophotometer\(^b\). The final concentration was determined by standard plate counts on TSA after 5 days incubation at 37° C and 5% CO\(_2\). For the preparation of killed vaccines, aliquots of heat inactivated 353-1 were adjusted to a concentration of 2.0 X 10\(^10\) CFU/ml in 0.15 M PBS and mixed with equal parts of a Quil A\(^c\) (1.0 mg/ml in 0.15 M PBS) or a squalene\(^d\)/Pluronic F127\(^d\) adjuvant which was prepared as previously described.\(^28\)

The challenge culture *B. suis* strain 3B (biovar 1) was prepared as previously reported.\(^22\) In order to assess the *in vitro* stability of the rough phenotype of 353-1, the culture was passaged weekly on TSA for the duration of experiment 2 for a total of 35 passages.

Swine experiments and study design
The pathogenicity and clearance of 353-1 were evaluated in weaned swine (experiment 1). A total of 21, 6 week old, cross-bred domestic swine were inoculated conjunctivally with 50 μl of 5.0 × 10^7 CFU of 353-1 in 0.15 M PBS. Six age-matched swine were sham inoculated with 50 μl of 0.15 M PBS. Inoculated and control swine were housed separately in biolevel 3 large animal containment facility and fed ad lib.

Induction of an immune responses, clearance, and protection from virulent challenge were also assessed in cross-bred domestic swine (experiment 2). A total of 44 6 week old swine were inoculated intramuscularly in the right cervical area with 2.0 ml of 0.15 M PBS containing 2 × 10^{10} CFU of 353-1. An additional 30 age-matched swine were orally inoculated with an average of 5.0 × 10^{11} CFU of 353-1/animal which was top-dressed onto their normal ration prior to feeding. Adjuvanted, heat-killed 353-1 vaccines were evaluated each in 5 age-matched swine which were vaccinated in both the right and left cervical areas with 2.0 ml of either the Quil A or squalene/Pluronic F127 adjuvanted vaccines which contained an equivalent of 2.0 × 10^{10} CFU of 353-1. Nine age-matched swine served as controls and were sham inoculated with 2.0 ml of 0.15 M PBS in the right cervical area. In addition, 4 age-matched, nonvaccinated swine were commingled with the parenterally vaccinated group to serve as sentinels to detect shedding of 353-1. All animals in experiment 2 were challenged at approximately week 30 after vaccination for the oral and parenteral group and week 16 after vaccination for the adjuvanted, killed vaccine groups by conjunctival administration of 5.0 × 10^7 CFU (volume=50 μl) of \textit{B. suis} 3B. The colonial phenotype was determined by direct observation and crystal violet staining.\textsuperscript{29}

**Serological Evaluation**

Blood was collected for serologic evaluation by cranial vena cava puncture in experiment 2 at weeks 0, 2, 4, 6, 8, 12, 14, 16, 18, 20, 24, 28, and 30 after vaccination and weeks 1, 2, 3, and 4 after challenge for the orally vaccinated and control groups; weeks 0, 3, 5, 7, 9, 11, 13, 17, 21, 25, 29, and 30 after vaccination and weeks 1, 2, 3, and 4 after challenge for the parenterally vaccinated and control groups; and weeks 0, 2, 4, 6, 8, 12, 14, and 16 after vaccination and weeks 1, 2, 3, and 4 after challenge for the killed vaccine and controls groups. After collection, blood samples were allowed to clot and
serum was separated by centrifugation. Serum was divided into 1 ml aliquots, and stored at -70°C until assays were performed. Seroconversion to 353-1 was determined by a microtiter plate ELISA assay. *B. suis* 353-1 used in ELISA assays was inactivated by treatment with 0.5% neutral buffered formalin for 24 h. at 4°C. Ninety-six well plates were coated with 100 μl of 1.0 X 10^10 CFU/ml of formalin-inactivated 353-1 in 0.1 M sodium carbonate/sodium bicarbonate buffer (pH 9.6) overnight at 4°C. After discarding the coating solution, plates were then blocked with 100 μl of 0.55% fish gelatin in 0.15 M phosphate buffered saline (PBS) at room temperature for 2 h. Plates were then washed 8 times with 300 μl of 0.15 M PBS containing 0.05% Tween 20 (PBS-T, pH 7.4). Plates were then coated with 100 μl of test sera at a dilution of 1:100 in PBS containing 0.55% fish gelatin. All samples were run in quadruplicate. After incubation for 2 h. at room temperature, test sera was discarded and plates were washed 8 times with 300 μl of PBS-T. Plates were then incubated with 100 μl of peroxidase-labeled rabbit anti swine IgG^d^ at a dilution of 1:500 in PBS containing 0.55% fish gelatin. After a 2 h. incubation at room temperature, the secondary antibody solution was discarded and plates were washed 8 times with 300 ml of PBS-T. Colorimetric reactions were developed with 0.0075% 3, 3', 5, 5'-tetramethylbenzidine in 0.10 M citrate buffer containing 0.015% hydrogen peroxide for 30 min. at room temperature. Reactions were stopped by the addition of 100 μl of 0.18 M sulfuric acid, and the optical density was read using an automated spectrophotometric plate at 450nm and 550 nm. The optical density at 550 nm was subtracted from the optical density at 450 nm and the 4 resulting values from each sample were averaged prior to statistical analysis.

Seroconversion to the *B. suis* challenge strain was determined by standard tube agglutination and card agglutination assays by previously described methods. Blood was collected on day 0 of challenge and weeks 1, 2, 3, and 4 after challenge from all swine.

**Peripheral blood mononuclear cell proliferation assays**

Blood (45 ml) was obtained in acid-citrate dextrose solution from the cranial vena cava at weeks 0, 6, 10, 14, and 18 after intraconjunctival inoculation with 353-1 from animals in experiment 1. In experiment 2 blood was similarly obtained at the following
Peripheral blood mononuclear cells (PBMCs) were enriched by density centrifugation using a Ficoll-sodium diatrizoate gradient. Fifty μl of each cell suspension containing 5.0 X 10^5 peripheral blood mononuclear cells were added to each of two separate flat-bottom wells of 96-well microtiter plates that contained 100 μl of RPMI 1640 medium only, 1640 medium containing 1.0 X 10^8 cells of heat-killed 353-1, or 1640 medium containing 1.2 μg of pokeweed mitogen (PWM). Cell cultures were incubated for 3 days at 37°C under 5% CO_2. The plates were then pulsed with 1.0 mCi of [³H] thymidine per well for 18 hours. Cells were harvested onto glass filter mats and counted for radioactivity in a liquid scintillation counter. Radioactivity was expressed as mean counts per minute (cpm), and stimulation indexes were obtained by dividing the mean cpm from antigen stimulated (1640 medium plus heat-killed 353-1) cells by the mean cpm from the nonstimulated (1640 medium only), control cell wells.

**Cytokine Assays**

In order to assess cytokine production of antigen stimulated PBMCs, duplicate plates to those prepared for proliferation assays were incubated for 4 days at 37°C under 5% CO_2. Plates were then frozen at -20°C until cytokine ELISAs were performed. A commercially available ELISA was used according to manufacturer’s instruction to assay interferon-gamma (IFN-γ) production of cultured PBMCs in experiment 2 at the following time points: weeks 0, 2, 4, 8, 10, 12, 14, 16, 20, 22, 24, 26, 28, and 30 after vaccination as well as weeks 1, 2, and 3 after virulent challenge for controls and orally vaccinated animals; weeks 0, 3, 5, 7, 8, 11, 15, 17, 19, 21, 23, 25, 27, 29, and 30 after vaccination and weeks 1, 2, and 3 after virulent challenge for controls and parenterally vaccinated animals; and weeks 0, 2, 6, 8, 10, 12, 14, and 16 after vaccination as well as weeks 1 and 3 after virulent challenge for controls and animals vaccinated with adjuvanted killed preparations. Interleukin-4 (IL-4) was also assayed from the same culture supernatant using a commercially available ELISA at weeks 0, 2, 6, 10, 14, and
for controls as well as oral and killed vaccine groups and weeks 0, 3, 7, 11, 15, and 17 for the control and parenteral group. Concentrations of the respective cytokines from the culture supernatants were determined by a log-log regressions analysis obtained from standard concentrations of IL-4 or IFN-γ. The concentration of the respective cytokines in the nonstimulated culture supernatant was subtracted from the antigen stimulated culture supernatant and means of the resulting differences were used to compare cytokine concentrations between controls and treatment groups.

**Necropsy**

All swine were euthanized by intra-cranial vena caval administration of sodium pentobarbital. Tissues collected for bacteriologic culture were collected using aseptic technique, placed into individual containers, and immediately frozen at -70°C until processed. Tissues collected for histologic evaluation were immediately placed in neutral-buffered 10% formalin, processed by routine paraffin embedding techniques, cut in 4 μm sections, and stained with hematoxylin and eosin.

In experiment 1, 2 inoculated pigs were necropsied at weeks 1, 2, and 3 after inoculation. Three inoculated pigs were necropsied at week 4 after inoculation. Five pigs were necropsied at weeks 10 and 14 after inoculation, and 8 inoculated pigs and the 6 control pigs were necropsied at week 18 after inoculation. Blood and urine were collected for bacteriologic culture at necropsy. The following tissues were collected for bacteriologic culture and histologic analysis: lung, liver, spleen, kidney, uterus, testis, seminal vesicle, bulbourethral gland, and prostate as well as medial retropharyngeal, mandibular, parotid, prescapular, prefemoral, popliteal, inguinal, iliac, ileocecal, sternal, and gastric hepatic lymph nodes.

In experiment 2, 4 orally vaccinated pigs were necropsied at weeks 4, 8, 16, 20, and 24 after vaccination. Four parenterally vaccinated pigs were necropsied at weeks 2 and 4 weeks after vaccination. Five parenterally vaccinated pigs were necropsied at weeks 6, 8, and 12 after vaccination, and 4 parenterally vaccinated pigs were necropsied at weeks 16, 20, and 24 after vaccination. Nine control pigs, 10 orally vaccinated pigs, 9 parenterally vaccinated pigs, 4 pigs which were sentinels to the parenterally vaccinated group, and 10 pigs vaccinated with the adjuvanted, killed vaccines were necropsied 30-32
days after virulent challenge. Whole blood, and urine were taken for culture, and lung, liver, spleen, kidney, uterus, testis, seminal vesicle, bulbourethral gland, and prostate as well as tracheobronchial, prescapular, medial retropharyngeal, mandibular, parotid, sternal, popliteal, and gastrohepatic, lymph nodes were taken for bacteriologic culture and histology.

**Bacteriologic culture**

In Experiment 1 whole blood as well as conjunctival, nasal, and vaginal swabs were collected for bacteriologic culture at day 0 and weeks 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, and 18 after infection. In Experiment 2 whole blood, conjunctival and nasal swabs, and environmental room swabs were collected for bacteriologic culture at day 0 and weekly after infection until necropsy. Whole blood and conjunctival and nasal swabs were collected at day 0 and weeks 1, 2, 3, and 4 after virulent challenge.

Whole blood for bacteriologic culture was immediately placed into tryptose broth containing acid citrate dextrose (1:1 v:v) and incubated for 7 days at 37°C and 5% CO₂ prior to plating on *Brucella* selective media. Urine was incubated in tryptose broth containing 5% bovine serum (1:3 v:v) for 7 days at 37°C and 5% CO₂ prior to plating on *Brucella* selective media. Environmental room swabs were incubated for 7 days at 37°C and 5% CO₂ in 300 ml of Kudzas Morse broth (tryptose base with 5% bovine serum, 25 U/ml bacitracin, 6 U/ml polymyxin B, 100 μg/ml cycloheximide, and 1.4 μg/ml ethyl violet). All other swabs were plated directly onto TSA and Kudzas Morse agar (KM; tryptose agar base with 5% bovine serum, 25 U/ml bacitracin, 6 U/ml polymyxin B, 100 μg/ml cycloheximide, and 1.4 μg/ml ethyl violet) and incubated for 7 days at 37°C and 5% CO₂. After thawing, tissues were individually ground in approximately 10% (w/v) sterile PBS (pH=7.2) using sterile glass grinders. Aliquots (100 μl) of each tissue homogenate or preincubated tryptose or KM broth (blood, urine, and environmental room swabs) were plated onto TSA and KM plates and were incubated at 37°C in 5% CO₂ for 7 days.

**Culture identification:**

*Brucella* suspect cultures were identified on the basis of colony morphology, growth characteristics, and growth on selective media. All suspect *Brucella* cultures
recovered were especially checked for smooth or rough colony morphology. Isolates were identified as *Brucella spp.* by a real time polymerase chain reaction technique (PCR) using *Brucella*-specific primers and probe to the omp2A region of the *Brucella* genome.\textsuperscript{30} Reactions consisted of 20 μl and contained 2 μl of suspect culture in tris-EDTA and 18 μl of reaction mixture consisting of 200 μM each of dATP, dCTP, dGTP, and dTTP, 1 X PCR Buffer II, 3.0 mM MgCl2, 1.25 U AmpliTaq Gold polymerase, 0.5 μM of each upstream (5’-CCCAAGCATTGTCTTCAGCAACAG-3’) and downstream (5’-TGGTCTGAAGTATCAGGCTACGCA-3’) primers, and 0.1 mM of probe (5’-ACCTTGGTGATAGAAACCTTCCGGCGT-3’) which was labeled with 6-FAM on the 5’ end and Black Hole Quencher 1 on the 3’ end. The primers had 100% conservancy within the genomes of *B. suis*, *B. abortus*, and *B. melitensis* according to BLAST analysis. Tris-EDTA and a culture of *Yersinia enterocolitica* O:9 served as negative controls, and *Brucella suis* strain 3B and 353-1 served as a positive control. Samples were cycled in a Rotor Gene 3000\textsuperscript{m} real time PCR thermal cycler under the following conditions: a single 10 minute activation at 95°C, followed by 40 cycles consisting of 60 sec. at 60°C and 20 sec. at 95°C. Mean fluorescence was recorded each cycle after the 60°C step. Thresholds were determined for each individual run using the positive control reactions, and test samples were considered positive if the mean change in fluorescence went above the threshold.

**Statistical Analysis**

For all statistical analyses a level of P<0.05 was used to determine differences between vaccinates and controls. Serologic response data to 353-1 were compared between vaccinates and controls using Student’s t-test\textsuperscript{n}. Stimulation indexes of proliferative responses to 353-1 were compared between vaccinated and controls using a general linear model procedure and least squared means\textsuperscript{o}. Fisher’s exact test\textsuperscript{p} was used to determine differences in culture positivity on a per tissue basis between vaccinates and controls. Bacterial culture concentrations from tissues which were quantified were converted to the logarithm of the resulting CFU/g and compared between vaccinates and controls using Student’s t-test\textsuperscript{n}. Fisher’s Exact Test\textsuperscript{p} was also used to determine differences between vaccinates and controls for histologic lesions.
Results

Bacterial Cultures

In experiment 1, plate counts yielded a culture concentration of $7.3 \times 10^8$ CFU/ml of 353-1 which equaled a per animal dosage of $3.65 \times 10^7$ CFU. In experiment 2 plate counts of 353-1 yielded culture concentrations of $0.97 \times 10^{10}$ CFU/ml which equaled a per animal dosage of $1.94 \times 10^{10}$ CFU for parenteral vaccinates and $1.46 \times 10^{11}$ CFU for oral vaccinates. Plate counts for the challenge strain *B. suis* 3B yielded a culture concentration of $6.7 \times 10^8$ CFU/ml which equaled a per animal dosage of $3.35 \times 10^7$ CFU. All passages (n=35) of 353-1 produced cultures with rough phenotype colonies as observed by direct observation and by crystal violet colony staining.

Serological evaluation

There were no significant differences in Experiment 1 between 353-1 inoculated pigs and controls on 353-1 ELISAs. All sera samples in Experiment 1 were also negative by the *Brucella* card test and STT serological assays. The results of 353-1 ELISAs in Experiment 2 are given in Figure 1 for parenterally vaccinated pigs and Figure 2 for pigs vaccinated with adjuvanted. Briefly, there were statistically significant differences between controls and parenterally vaccinated pigs as well as pigs vaccinated with adjuvanted, heat-killed vaccines at certain weeks after vaccination; however, there were no significant differences between controls and orally vaccinated pigs. All prechallenge, postvaccination sera samples in Experiment 2 were also negative by the *Brucella* card and STT serologic assays.

Peripheral blood mononuclear cell proliferation assays

The results of PBMC proliferation assays from Experiment 1 are shown in Figure 3. Inoculated pigs had significantly higher stimulation indexes at weeks 10 and 18 after inoculation. All vaccine preparations induced significant PBMC proliferation from vaccinated animals compared to controls. Parenterally vaccinated animals had significantly higher stimulation indexes compared to controls at weeks 7, 9, 11, 15, 17, 19, 21, 23, 25, 27, 29, and 30 (Figure 4). Orally vaccinated animals had significantly higher stimulation indexes compared to controls at weeks 2, 4, 6, 8, 14, 16, 18, 20, 22,
Pigs vaccinated with heat-killed 353-1 adjuvanted with squalene/Pluronic F127 had significantly higher stimulation indexes at weeks 6, 8, 10, and 14 compared to controls, and animals in the Quil A adjuvant group had significantly higher stimulation indexes at weeks 4, 6, 8, and 10 compared to controls (Figure 6).

**Cytokine ELISAs**

In Experiment 2 there was no significant IL-4 production in 353-1 antigen stimulated PBMCs from vaccinated animals compared to controls at all weeks assayed. PBMCs from parenterally vaccinated pigs had significantly higher IFN-γ production at weeks 3, 5, 7, 15, 17, 21, 29, and 30 after vaccination as well as weeks 1 and 2 after challenge compared to controls (Figure 7). IFN-γ production was significantly higher at weeks 8, 16, 20, 22, 26, 28, and 30 after vaccination as well as week 1 after challenge in the orally vaccinated group compared to the control group (Figure 8). In the adjuvanted, heat-killed vaccine groups, only the squalene/Pluronic F127 preparation induced significant IFN-γ production at weeks 8 and 10 after vaccination (Figure 9).

**Distribution, shedding, clearance, and pathogenicity of 353-1**

Table 1 lists the postinoculation, antemortem culture results of Experiment 1. *B. suis* 353-1 was only isolated from the blood (n=7) and conjunctival swabs (n=2). In Experiment 2, 353-1 was only isolated from blood (Table 2) from both parenteral (n=30) and oral (n=26) vaccinates. Weekly environmental swabs from the rooms housing the parenteral and oral vaccine groups in Experiment 2 yielded no growth of 353-1 at all weeks. Bacterial culture results from necropsy samples from Experiment 1 (Table 3) and postvaccination, prechallenge necropsy samples from Experiment 2 (Tables 4 and 5) showed that 353-1 was first isolated from draining lymph nodes followed by isolation from peripheral lymphoreticular tissues. All postinoculation isolates from Experiment 1 and postvaccination, prechallenge isolates from Experiment 2 exhibited the rough phenotype by direct observation and crystal violet staining of all colonies examined. Most isolations were clustered between weeks 1 to 12; however, a single animal was positive at week 18 after inoculation in Experiment 1. In Experiment 2 there were no bacterial culture isolations of 353-1 at all time points from the 4 sentinel pigs commingled with the parenteral vaccinates. In Experiment 1 there were no significant
histological lesions in inoculated animals compared to controls. In Experiment 2 mild, multifocal lymphoplasmacytic interstitial nephritis was the only histological lesion noted in postvaccination, prechallenge necropsy samples. Small aggregates of small lymphocytes and lesser numbers of plasma cells were present between tubules, mostly in the cortex, and adjacent to some glomeruli in 1 oral vaccinate at 4 weeks after vaccination and in 4 parenteral vaccinates, one each at 4, 6, 16, and 24 weeks after vaccination.

**Postvaccination challenge studies**

Postchallenge serology and postchallenge, antemortem culture results for Experiment 2 are listed in Table 6. All animals seroconverted to *Brucella* as demonstrated by the *Brucella* card and STT assays; however seroconversion was delayed in all vaccine groups. All *B. suis* isolates from postchallenge swine from all groups at all time points were smooth as examined by direct observation and crystal violet staining. Postchallenge blood culture isolation of smooth *B. suis* was also lower in parenteral, oral and squalene groups, however, there was no statistical significance. Swab cultures (conjunctival and nasal) were lower in parenteral and oral groups compared to nonvaccinated controls. Table 7 lists the percentage of animals culture positive for smooth *B. suis* for each vaccine treatment group for each tissue examined. There was a statistically lower percentage of positive isolations from the spleen for oral and parenteral vaccinates and the medial retropharyngeal lymph node for oral vaccinates. Parenteral vaccinates also had significantly lower recovery of smooth *B. suis* on a CFU/g of tissue basis for the spleen and gastrohepatic, mandibular, and parotid lymph nodes (Figure 10). Likewise, there was significantly lower recovery of smooth *B. suis* on a CFU/g of tissue basis for oral vaccinates from the spleen and gastrohepatic, mandibular, medial retropharyngeal, and parotid lymph nodes (Figure 11). From both the squalene/Pluronic F127 and Quil A adjuvant groups, there was a significantly lower recovery of smooth *B. suis* from the spleen on a CFU/g of tissue basis (Figure 12).

Table 8 summarizes histological lesions found in all treatment groups after challenge with *B. suis* 3B. There was a significant difference in the number of animals with no significant lesions in the orally vaccinated and Quil A adjuvant groups compared
to nonvaccinated controls. There was also a significant difference in the number of animals exhibiting hepatitis in the orally vaccinated groups compared to controls. Hepatitis in affected animals was characterized by portal to random distribution of aggregates of lymphocytes and plasma cells with lesser numbers of neutrophils (Figure 13A). Other histological lesions found in control and vaccine groups after challenge included purulent to pyogranulomatous lymphadenitis (Figures 13B & 13C) and interstitial nephritis (13D). A single nonvaccinated, sentinel animal developed bilateral prostatitis and seminal vesiculitis characterized by a marked increase in space between ductules due to marked fibrosis and high numbers of lymphocytes, plasma cells, and neutrophils. Ductular lumena were also markedly dilated due to large numbers of neutrophils and sloughed, necrotic epithelial cells. There was segmental denudement of epithelial cells from the ductular epithelial surfaces and segmental epithelial hyperplasia.

**Discussion**

The studies reported here examined *B. suis* 353-1, a natural, rough mutant, with the goal of determining its potential as a vaccine candidate. The studies were designed to determine 1) if 353-1 is pathogenic to swine, 2) the clearance time of 353-1 in swine when given as a parenteral or oral vaccine 3) the immunogenicity of 353-1 and its ability to determine a cell-mediated immune response, and 4) the ability of 353-1 to protect from virulent *B. suis* challenge. *B. suis* 353-1 was examined in live vaccine preparations which were considered to be applicable to both domestic (parenteral administration) or feral (oral administration) swine. In addition, heat-killed preparations of 353-1 were also examined in adjuvant preparations of squalene/Pluronic F127 and Quil A which have been shown to enhance type 1-T helper lymphocyte (Th-1) cytokine responses.28, 31

Rough strains of *Brucella* are attractive as vaccine strains due to their general lack of virulence and their inability to cause seroconversion to standard *Brucella* serological assays.27, 32, 33 *B. suis* 353-1 induced no lesions when administered at a low dose (Experiment 1). Lymphoplasmacytic interstitial nephritis was the only histological lesion observed in swine administered a vaccine dose either orally or parenterally (Experiment 2). The results of these studies showed that 353-1 first colonized draining lymph nodes
and then was systemically distributed throughout the lymphoreticular system. Total clearance of 353-1 appears to be achieved by 20 weeks after inoculation/vaccination as the latest tissue isolation of 353-1 was at 18 weeks postinoculation in Experiment 1, and the last blood culture isolation of 353-1 was week 17 after parenteral vaccination. In addition no swine which were inoculated with a low dose by conjunctival administration or vaccine dose of 353-1 by parenteral or oral administration developed positive serological reactions to standard *Brucella* serological detection assays which rely on detecting antibodies to the O-polysaccharide side chain of LPS.

*B. suis* 353-1 also appears to not be shed by vaccinated animals. There was no isolation of 353-1 from weekly environmental swabs. Also, there were no nasal or conjunctival swabs culture positive for 353-1 after vaccination. In addition, 4 nonvaccinated swine were used as sentinel animals which were commingled with the group parenterally vaccinated with live 353-1. These animals were commingled from day 0 of vaccination, and none of these sentinels were blood culture positive for 353-1 at any of the weekly postvaccination sampling times.

Proliferation of antigen-stimulated PBMCs from postvaccination animals has been associated with protective immunity with *B. abortus* RB51 in both cattle and bison. Parenteral and oral vaccination with live 353-1 as well as vaccination with adjuvanted, heat-killed 353-1 induced proliferative responses in cultured PBMCs which were stimulated with heat-killed 353-1 antigen. These responses occurred sooner and were more robust than those associated with the lower dosage of 353-1 administered via conjunctival administration in Experiment 1.

IFN-γ has also been reported to be necessary for clearance of *B. abortus* in murine models, and IFN-γ production by antigen stimulated lymphoid cells has also been associated with protective immunity in *B. abortus* murine models. In swine, IFN-γ gene transcription has been found to be associated with intracellular *Toxplasma gondii* infection and has been recognized as a key marker of a Th-1 polarized immune response. In the current study, IFN-γ was significantly elevated at multiple time points after vaccination in the parenterally and orally vaccinated groups as well as the squalene/Pluronic F127 adjuvant group. There was also a lack of production of
interleukin-4 (IL-4) by the same antigen stimulated PBMCs. IL-4 upregulation has been associated with Th-2 polarized cell-mediated immune processes in swine.\textsuperscript{37} Murine models of brucellosis suggest that the lack of an IL-4 response is advantageous in allowing maximal production of Th-1 cytokines\textsuperscript{39} and in the clearance of \textit{Brucella abortus}\textsuperscript{40,41}

Humoral immune responses were also significantly higher in swine parenterally vaccinated with live 353-1 or adjuvanted heat killed 353-1. The orally vaccinated group had no differences in humoral responses to 353-1 as measured by ELISA compared to the control group. Although humoral responses are commonly observed against \textit{Brucella} vaccine strains which induce protective immunity,\textsuperscript{34,42} humoral immune responses themselves are not necessarily a correlate of protection in \textit{Brucella} vaccine models.\textsuperscript{43}

\textit{B. suis} 353-1 conferred partial protection to challenge with the virulent strain \textit{B. suis} 3B. Protection was assessed in these studies by bacterial recovery. Decreases in recovery of \textit{B. suis} 3B was most noteworthy in the groups parenterally and orally vaccinated with live 353-1 in these groups there was a significant decrease in the log CFU/g of tissue recovery of \textit{B. suis} 3B in multiple tissues. There was also a significant decrease in the number of animals being splenic culture positive from those two groups and a decrease in the number of animals which were culture positive from the medial retropharyngeal lymph node in the orally vaccinated group. Both adjuvant groups also had a significant decrease in recovery of \textit{B. suis} 3B from the spleen on a log CFU/g of tissue basis. Antemortem, postchallenge recovery of \textit{B. suis} 3B was significantly lower 1 week after challenge in both live vaccine groups. All vaccinated groups also exhibited a delay in seroconversion to \textit{B. suis} 3B by one week compared to controls.

Histological lesions consistent with those previously reported for \textit{B. suis}\textsuperscript{5} were present in postchallenge necropsy samples. Although there was a decrease in lesions in all vaccine groups, there was a statistically significant decrease in total lesions in the orally vaccinated group and the group vaccinated with Quil A adjuvanted heat-killed 353-1 and a statistically significant decrease in hepatitis in the orally vaccinated group.

The results of these studies suggest that 353-1 is a stable, rough mutant of \textit{B. suis} which may have promise in the prevention of swine brucellosis. \textit{B. suis} 353-1 was shown
to induce a Th-1 polarized immune response in vaccinated swine and confer partial protection to challenge with virulent *B. suis*. The preliminary studies reported here suggest that further investigations into applications of 353-1 could be beneficial to develop tools for aiding efforts in controlling swine brucellosis in domestic and feral populations.

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Names are necessary to report factually on available data. Mention of trade names or commercial products in the article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture.

*a*Difco Laboratories, Detroit, MI, USA.

*b*Beckman, Palo Alto, CA, USA.

*c*Accurate Chemicals, Westbury, NY, USA.

*d*Sigma, St. Louis, MO, USA.

*e*Immulux, Dynex, Chantilly, VA, USA.

*f*Vmax Kinetic Microplate Reader, Molecular Devices, Sunnyvale, CA, USA.

*g*Histopaque 1083, Sigma, St. Louis, MO, USA.

*h*1450 Microbeta Scintillation Counter, Wallac, Inc., Gaithersburg, MD, USA.

*i*Swine IFN-g Cytoset, Biosource, Camarillo, CA, USA.

*j*Swine IL-4 Cytoset, Biosource International, Camarillo, CA, USA.

*k*Boehringer Mannheim, Indianapolis, IN, USA.

*l*Perkin Elmer, Branchburg, NJ, USA.

*m*Corbett Life Science, Sydney, Australia.

*n*PROC MEANS T, SAS Statistical Software, SAS Institute, Inc., Cary, NC, USA.
PROC MEANS LSMEANS, SAS Statistical Software, SAS Institute, Inc., Cary, NC, USA.

PROC FREQ CHISQ, SAS Statistical Software, SAS Institute, Inc., Cary, NC, USA.

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37. Dawson HD, Beshah E, Nishi S, et al. Localized multigene expression patterns support an evolving Th1/Th2-like paradigm in response to infections with *Toxoplasma gondii* and *Ascaris suum*.


Table 1. Experiment 1, postinoculation, antemortem recovery of \textit{B. suis} 353-1. Twenty-one 6 week old, crossbred domestic swine were conjunctivally inoculated with $5.0 \times 10^7$ \textit{B. suis} 353-1 and sampled weekly 1 to 18 weeks after inoculation.

<table>
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<th>2</th>
<th>3</th>
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<td>0/7</td>
<td>0/2</td>
<td>0/7</td>
</tr>
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</table>

*There were no positive samples in any groups weeks 4 to 17 after inoculation.

Table 2. Experiment 2, postvaccination, antemortem blood culture recovery of \textit{B. suis} 353-1. Six week old, crossbred, domestic swine were either parenterally vaccinated with $2.0 \times 10^{10}$ CFU of \textit{B. suis} 353-1 or orally vaccinated with an average dose of $5.0 \times 10^{11}$ \textit{B. suis} 353-1.

<table>
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<tr>
<th>Week after vaccination</th>
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<th>3</th>
<th>4</th>
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<th>7</th>
<th>8</th>
<th>9</th>
<th>17*</th>
<th>Total</th>
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<td>1/26</td>
<td>1/17</td>
<td>30/44</td>
<td></td>
</tr>
<tr>
<td>Oral Vaccinates</td>
<td>18/30</td>
<td>17/30</td>
<td>2/30</td>
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<td>1/26</td>
<td>0/22</td>
<td>0/22</td>
<td>26/30</td>
</tr>
</tbody>
</table>

*No animals were blood culture positive for \textit{B. suis} 353-1 between weeks 9 and 17 or after week 17 after vaccination.
Table 3. Experiment 1, postinoculation recovery of \textit{B. suis} 353-1. A total of 21 crossbred, domestic swine were conjunctivally inoculated with 5.0 $\times$ 10$^7$ CFU \textit{B. suis} 353-1 and necropsied 1-18 weeks after inoculation.

<table>
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</tr>
<tr>
<td>Liver</td>
<td>0/2</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/2</td>
</tr>
<tr>
<td>Kidney</td>
<td>0/2</td>
</tr>
<tr>
<td>Medial Retropharyngeal LN</td>
<td>1/2</td>
</tr>
<tr>
<td>Mandibular LN</td>
<td>0/2</td>
</tr>
<tr>
<td>Parotid LN</td>
<td>0/2</td>
</tr>
<tr>
<td>Prescapular LN</td>
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<tr>
<td>Prefemoral LN</td>
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</tr>
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<td>Popliteal LN</td>
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<td>Inginal LN</td>
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</tr>
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<td>Ileocecal LN</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Tracheobronchial LN</td>
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<tr>
<td>Gastrohepatic LN</td>
<td>0/2</td>
</tr>
<tr>
<td>Uterus</td>
<td>NA</td>
</tr>
<tr>
<td>Testis</td>
<td>0/2</td>
</tr>
<tr>
<td>Male Accessory Sex Glands</td>
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</tr>
<tr>
<td>Urine</td>
<td>0/2</td>
</tr>
<tr>
<td>Total number of swine culture positive</td>
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</tr>
</tbody>
</table>
Table 4. Experiment 2, postvaccination recovery of *B. suis* 353-1 from samples obtained at necropsy from crossbred, domestic swine parenterally vaccinated with $2.0 \times 10^{10}$ CFU of *B. suis* 353-1.

<table>
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<td>0/4</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0/4</td>
<td>1/4</td>
<td>0/5</td>
<td>1/5</td>
<td>0/5</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/4</td>
<td>1/4</td>
<td>0/5</td>
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<td>0/4</td>
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</tr>
<tr>
<td>Kidney</td>
<td>0/4</td>
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<td>0/5</td>
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<td>0/4</td>
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<tr>
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</tr>
<tr>
<td>Prescapular LN</td>
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<td>0/4</td>
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<tr>
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<td>0/5</td>
<td>0/4</td>
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</tr>
<tr>
<td>Tracheobronchial LN</td>
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<td>1/4</td>
<td>1/5</td>
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<td>0/5</td>
<td>0/4</td>
<td>0/4</td>
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</tr>
<tr>
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<td>0/2</td>
<td>0/2</td>
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<td>0/3</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
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<td>0/2</td>
<td>0/3</td>
<td>0/2</td>
<td>0/2</td>
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</table>
Table 5. Experiment 2, postvaccination recovery of *B. suis* 353-1 from samples obtained at necropsy from crossbred, domestic swine orally vaccinated with an average dose of 5.0 X 10^{11} CFU of *B. suis* 353-1.

<table>
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</tr>
<tr>
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</tr>
<tr>
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<td>0/4</td>
</tr>
<tr>
<td>Kidney</td>
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<tr>
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<tr>
<td>Medial Retropharyngeal LN</td>
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<tr>
<td>Prescapular LN</td>
<td>0/4</td>
</tr>
<tr>
<td>Parotid LN</td>
<td>0/4</td>
</tr>
<tr>
<td>Popliteal LN</td>
<td>0/4</td>
</tr>
<tr>
<td>Sternal LN</td>
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<tr>
<td>Tracheobronchial LN</td>
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<tr>
<td>Uterus</td>
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<tr>
<td>Testis</td>
<td>0/2</td>
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<tr>
<td>Urine</td>
<td>0/4</td>
</tr>
<tr>
<td>Total number of swine culture positive</td>
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</table>
Table 6. Postchallenge seroconversion and antemortem bacterial culture results of control swine and swine vaccinates with B. suis 353-1. Swine were conjunctivally challenged with 5.0 X 10^7 CFU of B. suis 3B 30 weeks (parenteral and oral groups) or 16 weeks (adjuvanted, heat-killed vaccine groups) after vaccination.

<table>
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<tr>
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<td>1/9</td>
<td>0/9</td>
<td>5/9</td>
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<td>0/9</td>
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<td>3/9</td>
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<td>0/10</td>
<td>0/10</td>
<td>2/10</td>
</tr>
<tr>
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<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>1/5</td>
</tr>
<tr>
<td>Quil A Vaccinates</td>
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<td>3/5</td>
<td>2/5</td>
<td>5/5</td>
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<td><strong>Swab Culture</strong></td>
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<tr>
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<td>2/9</td>
<td>0/9</td>
<td>0/9</td>
<td>5/9</td>
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<td>0/10</td>
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<td>1/10*</td>
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<td>2/5</td>
<td>2/5</td>
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<tr>
<td>Quil A Vaccinates</td>
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<td>1/5</td>
<td>3/5</td>
<td>2/5</td>
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</tr>
</tbody>
</table>

*P<0.05
Table 7. Experiment 2, postchallenge recovery of *B. suis* 3B. Results are expressed as the percentage of animals from which *B. suis* 3B was recovered from controls (n=9), pigs orally vaccinated with an average dose of 5.0 $\times 10^{11}$ *B. suis* 353-1 (n=10), pigs intramuscularly vaccinated with 2.0 $\times 10^{10}$ CFU *B. suis* 353-1 (n=9), and pigs intramuscularly vaccinated with 2.0 $\times 10^{10}$ CFU of heat-killed *B. suis* 353-1 which was adjuvanted with squalene/Pluronic F127 (n=5) or Quil A (n=5). Animals were challenged with 5.0 $\times 10^{7}$ CFU *B. suis* 3B via conjunctival administration 30 weeks (oral and parenteral groups) or 16 weeks (heat-killed vaccine groups) after vaccination.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Controls</th>
<th>Oral Vaccinates</th>
<th>Parenteral Vaccinates</th>
<th>Squalene Vaccinates</th>
<th>Quil A Vaccinates</th>
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</thead>
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</tr>
<tr>
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<td>10.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
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<td>11.1</td>
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</tr>
<tr>
<td>Liver</td>
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</tr>
<tr>
<td>Spleen</td>
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<td>0.0*</td>
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<tr>
<td>Kidney</td>
<td>11.1</td>
<td>0.0</td>
<td>0.0</td>
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<td>100</td>
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<td>60.0</td>
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<td>100</td>
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<td>80.0</td>
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<td>60.0</td>
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<td>44.4</td>
<td>60.0</td>
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</table>

*P<0.05
Table 8. Summary of histological lesions by treatment group observed after challenge with 5.0 X 10^7 CFU *B. suis* 3B via conjunctival route 30 weeks (parenteral and oral group) or 16 weeks (squalene and Quil A group) after vaccination.

<table>
<thead>
<tr>
<th></th>
<th>Hepatitis(^1)</th>
<th>Lymphadenitis(^2)</th>
<th>Interstitial Nepritis(^3)</th>
<th>Inflammation of Male Accessory Sex Gland(^4)</th>
<th>NSL(^5)</th>
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</thead>
<tbody>
<tr>
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<td>0/9</td>
<td>2/9</td>
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<tr>
<td>Sentinel</td>
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<td>2/4</td>
<td>0/4</td>
<td>1/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Parenteral</td>
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<td>1/9</td>
<td>1/9</td>
<td>0/9</td>
<td>6/9</td>
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<tr>
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<td>0/10*</td>
<td>0/10</td>
<td>1/10</td>
<td>0/10</td>
<td>9/10*</td>
</tr>
<tr>
<td>Squalene</td>
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<td>0/5</td>
<td>2/5</td>
<td>0/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Quil A</td>
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<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>5/5*</td>
</tr>
</tbody>
</table>

\(^1\) Multifocal, lymphoplasmacytic and purulent portal to random hepatitis  
\(^2\) Multifocal to locally extensive purulent to pyogranulomatous lymphadenitis  
\(^3\) Multifocal, lymphocytic interstitial nephritis  
\(^4\) Diffuse, lymphoplasmacytic and purulent prostatitis and seminal vessiculitis with marked fibrosis  
\(^5\) No significant lesions  
*P<0.05
Figure 1. Serologic responses of negative control pigs (dark boxes, n=9) and pigs intramuscularly vaccinated with $2.0 \times 10^{10}$ CFU *B. suis* 353-1 (light boxes, n=9) to heat-killed *B. suis* 353-1 ELISAs. Responses are reported as mean optical density (OD) ± SEM.

*P<0.05
Figure 2. Serologic responses of negative control pigs (dark boxes, n=9) and pigs intramuscularly vaccinated with $2.0 \times 10^{10}$ CFU of heat-killed *B. suis* 353-1 adjuvanted with either squalene/Pluronic F127 (light boxes, n=5) or Quil A (open boxes, n=5) to heat-killed *B. suis* 353-1 ELISAs. Responses are reported as mean optical density (OD) ± SEM.

$^{a}$P<0.05 squalene/pluronic F127 adjuvant group  
$^{b}$P<0.05 Quil A adjuvant group
Figure 3. Experiment 1, peripheral blood mononuclear cell (PBMC) blastogenesis assays of negative control pigs (dark boxes) and pigs conjunctivally inoculated with 5.0 X 10^7 CFU *B. suis* 353-1. Proliferative responses are expressed as stimulation indexes ± SEM. Data was obtained from 6 (week 18) or 10 (weeks 0, 6, 10, and 15) inoculated pigs and 6 control pigs per time period.

*P<0.05
Figure 4. Experiment 2, peripheral blood mononuclear cell (PBMC) blastogenesis assays of parenterally vaccinated (light boxes) and sham vaccinated controls (dark boxes). Proliferative responses obtained after intramuscular vaccination with $2.0 \times 10^{10}$ CFU *B. suis* 353-1. Results are expressed as stimulation indexes ± SEM. Data was obtained from 9 vaccinates and 9 control animals per time period.

*P<0.05
Figure 5. Experiment 2, peripheral blood mononuclear cell (PBMC) blastogenesis assays of orally vaccinated (light boxes) and nonvaccinated controls (dark boxes). Proliferative responses obtained after oral vaccination with an average dose of $5.0 \times 10^{11}$ CFU *B. suis* 353-1. Results are expressed as stimulation indexes ± SEM. Data was obtained from 10 vaccinates and 9 control animals per time period.

*P<0.05
Figure 6. Experiment 2, peripheral blood mononuclear cell (PBMC) blastogenesis assays of swine intramuscularly vaccinated with $2.0 \times 10^{10}$ CFU of heat-killed \textit{B. suis} 353-1. Proliferative responses of controls (dark boxes, n=9), pigs vaccinated with squalene/Pluronic F127 adjuvanted vaccine (light boxes, n=5), and Quil A adjuvanted vaccine (open boxes n=5) are expressed as stimulation indexes ± SEM.

\(^{a}\)P<0.05, squalene/Pluronic F127 adjuvant group
\(^{b}\)P<0.05, Quil A adjuvant group
Figure 7. Experiment 2, results of interferon-gamma (IFN-\(\gamma\)) ELISAs from culture supernatant of antigen-stimulated peripheral blood mononuclear cells (PBMCs) from controls (dark boxes, n=9) and pigs intramuscularly vaccinated with 2.0 \(\times\) 10^{10} CFU \textit{B. suis} 353-1. Results are expressed as mean IFN-\(\gamma\) concentration ± SEM.

\*\textit{P}<0.05
Figure 8. Experiment 2, results of interferon-gamma (IFN-γ) ELISAs from culture supernatant of antigen-stimulated peripheral blood mononuclear cells (PBMCs) from controls (dark boxes, n=9) and pigs orally vaccinated with an average dose of $5.0 \times 10^{11}$ CFU of *B. suis* 353-1 (light boxes, n=10). Results are expressed as mean IFN-γ concentration $\pm$ SEM.

*P<0.05
Figure 9. Experiment 2, results of interferon-gamma (IFN-γ) ELISAs from culture supernatant of antigen-stimulated peripheral blood mononuclear cells (PBMCs) from controls (dark boxes, n=9) and pigs intramuscularly vaccinated with 2.0 X 10^{10} CFU of heat-killed *B. suis* 353-1 adjuvanted with either squalene/Pluronic F127 (light boxes) or Quil A (open boxes). Results are expressed as mean IFN-γ concentration ± SEM.

*P<0.05, squalene/Pluronic F127 group.*
Figure 10. Experiment 2, postchallenge recovery of *B. suis* 3B from tissues obtained at necropsy 30-32 days after challenge with 5.0 X 10^7 CFU *B. suis* 3B. Bacterial recovery is expressed as log CFU/g of tissue ± SEM for controls (dark boxes, n=9) and pigs intramuscularly vaccinated with 2.0 X 10^{10} CFU of *B. suis* 353-1 (light boxes, n=9) 30 weeks prior to challenge.

*P<0.05.
Figure 11. Experiment 2, postchallenge recovery of *B. suis* 3B from tissues obtained at necropsy 30-32 days after challenge with 5.0 X 10^7 CFU *B. suis* 3B. Bacterial recovery is expressed as log CFU/g of tissue ± SEM for controls (dark boxes, n=9) and pigs orally vaccinated with an average dose of 5.0 X 10^{11} CFU of *B. suis* 353-1 (light boxes, n=10) 30 weeks prior to challenge.
Figure 12. Experiment 2, postchallenge recovery of *B. suis* 3B from tissues obtained at necropsy 30-32 days after challenge with $5.0 \times 10^7$ CFU *B. suis* 3B. Bacterial recovery is expressed as log CFU/g of tissue ± SEM for controls (dark boxes, n=9) and pigs intramuscularly vaccinated with $2.0 \times 10^{10}$ CFU of heat-killed *B. suis* 353-1 adjuvanted with either squalene/Pluronic F127 (light boxes, n=5) or Quil A (open boxes, n=5) 16 weeks prior to challenge.

*P<0.05*
Figure 13A. Portal hepatitis in a nonvaccinated, control animals 32 days after challenge with $5.0 \times 10^7$ CFU *B. suis* 353-1. The portal area is expanded with lymphocytes, plasma cells, and neutrophils which extend beyond the limiting plate.

Figure 13B. Purulent and histiocytic lymphadenitis in a nonvaccinated, control animal 32 days after challenge with $5.0 \times 10^7$ CFU *B. suis* 3B. Sinuses are expanded by large numbers of neutrophils and moderate numbers of macrophages.
Figure 13C. Granulomatous lymphadenitis in a nonvaccinated, control animal 32 days after challenge with $5.0 \times 10^7$ CFU *B. suis* 3B. The perifollicular area contains dense aggregates of multinucleated giant cells and epithelioid macrophages.

Figure 13D. Lymphoplasmacytic interstitial nephritis in a nonvaccinated, control animal 32 days after challenged with $5.0 \times \text{CFU} \ 10^7 \ B. \ suis \ 3B$. Aggregates of lymphocytes and plasma cells are found segmentally around a glomerulus and between cortical tubules.
CHAPTER 7. GENERAL CONCLUSIONS

Due to the near eradication of brucellosis in domestic livestock within the US, there has been increased interest in developing control efforts for brucellosis in wildlife reservoirs of Brucella. Feral swine, which are continually penetrating new areas in the US and growing in population, serve as a reservoir for Brucella in at least 15 states. USDA Animal and Plant Health Inspection Service (APHIS) and Wildlife Services (WS) have expressed great interest in utilizing vaccines to control brucellosis in feral swine populations. Early reports of immunogenicity and efficacy of the vaccine B. abortus RB51 (RB51)\textsuperscript{9,12} within the research and regulatory community prompted an effort to further characterize RB51 as well as other potential vaccine candidates. The work reported within this dissertation attempted to contribute to the knowledge base which will aid in dealing with feral swine brucellosis by characterizing Brucella infection and diagnostics within an enzootically feral swine population as well as characterizing candidate vaccines within feral and domestic swine.

The studies reported within Chapter 3 examined an isolated feral swine herd on an island on the Atlantic coast of South Carolina. This herd had been previously documented to be enzootically infected with Brucella based on serological assessments.\textsuperscript{10,23} It was found in the current study that this population is harboring multiple strains of both B. suis and B. abortus. Strain differences among the biovar 1 isolates of B. suis were found using an assay which assessed 8 loci of a variable nucleotide tandem repeat (VNTR) within the Brucella genome.\textsuperscript{3,4} A VNTR assay was also used to examine the B. abortus isolates; however, isolate differences were more pronounced as the B. abortus strain 19 (S19) vaccine was found to be enzootic within the population. This was the first report of a feral swine population enzootically infected with B. abortus. The vaccine strain RB51 was also found within the population. RB51 was introduced into the population as part of the studies reported in Chapter 4. The establishment of chronic infections with B. abortus was unexpected, as most experimental infection studies with B. abortus in swine have found that there is only a
short term infection of only several weeks duration of the lymph nodes which drains the site of infection.\textsuperscript{1, 8, 14, 15} Indeed studies with RB51 in domestic swine reported in Chapter 5 found that RB51 could only be isolated from the draining lymph node within a few weeks after experimental infection. No animals became bacteremic or systemically infected with RB51 in the domestic swine trials reported in Chapter 5. This interesting discrepancy between experimental infection and field observation has several possible explanations. First, the \textit{B. abortus} isolates from the feral swine could be swine adapted exhibiting particular tropism for swine tissues which enhances their infection. Experimental infections were conducted with these isolates in domestic swine (unpublished data), and the results were highly similar to other experimental infections with \textit{B. abortus} in swine; these isolates only established a short-lived infection of the draining lymph nodes. Second, perhaps prior infection with \textit{B. suis} allows enhancement of infection with \textit{B. abortus} in swine. This type of immune-mediated enhancement of infection has been reported with other species of antigenically similar bacteria.\textsuperscript{13, 21} Although many animals harbored both \textit{B. abortus} and \textit{B. suis}, \textit{B. abortus} was the only species isolated from seven of 62 culture positive animals. However, making individual species isolations in the face of a mixed \textit{Brucella} infection is tedious; therefore, the lack of a \textit{B. suis} culture does not totally exclude its presence, particularly within this herd with such a high culture prevalence of \textit{Brucella}. Another interesting possibility is the down regulation of a typical Th1 response by other pathogens. This herd had a very high prevalence rate of nematode infestation particularly from \textit{Stephanurus dentatus}. Helminth infestation has been shown to down regulate the Th1 response and enhance infection with bacteria.\textsuperscript{2, 5, 18, 20} However, further investigations into how this process may enhance \textit{Brucella} infections have not been undertaken.

The extremely high prevalence of \textit{Brucella} infection in this feral swine herd reiterates the predisposition which has been documented in the literature for swine to become chronically infected with \textit{B. suis}. This high culture positivity rate allowed some general assessments of serological assays to be made. The apparent sensitivity rate of the three assays investigated in these studies was quite low. When these tests were used in parallel, the overall sensitivity was 54.1\% and individual assay sensitivity rates ranged
from 13.1% to 44.6%. This is contrasted with the seroconversion rates observed in the experiments from Chapters 5 and 6. Using the same three assays in parallel, the sensitivity rate was 100% when sera from swine challenged with *B. suis* 3B were used. This extreme difference suggests that the sensitivity for these assays is excellent in the case of acute infection and extremely poor in the case of chronic infection and that these assays are unreliable for determining individual infected animals.

The results from the studies reported in Chapters 4 and 5 fail to support the immunogenicity and efficacy of RB51. Contrary to what was previously published, RB51 did not induce a cell-mediated or humoral immune response in vaccinated, domestic swine. Total clearance of RB51 was achieved quickly without first establishing a systemic distribution of the bacterium. This lack of immune responses and systemic distribution was associated with a lack of protection in a pregnant gilt challenge model based on bacterial recovery from maternal and fetal tissues. A field trial with RB51 in feral swine also had no effect on preventing further infection and seroconversion to *Brucella* within the vaccinated population. Furthermore, as previously mentioned, RB51 established a chronic infection in 13.6% of the recapture vaccinated animals. There were major differences in the study designs which could explain the incongruity among the results of the current study and those of the previously reported studies dealing with RB51 in swine. Within the Lord study, there was no defined challenge dose; gilts were bred to boars which were previously determined to be shedding *B. suis* in their semen. However, there was no attempt to validate and quantitated *Brucella* shedding rates within semen on the days of breeding; therefore, there was an undefined challenge dose. Furthermore, the criteria for determining protection centered on the abortion rate. Cultures from fetal tissues and gilt vaginal swabs were only performed on animals which were deemed to have aborted, which in the vaccine groups was 0%. As previously demonstrated in the literature, abortion is a relatively rare event in a *Brucella* infected swine herd particularly in chronically infected herds. Edmond’s study determined that swine vaccinated with RB51 developed significant humoral immune responses; however, there was no nonvaccinated, age-matched control group within that study. Therefore, that determination seems invalid.
The studies reported in Chapter 6 examined a naturally rough mutant of *B. suis*, *B. suis* 353-1 (353-1) as a vaccine candidate in swine. The original isolate of 353-1 was made from the urine of a feral pig; therefore, there was initial skepticism on the potential for it to be a vaccine because of the possibility of urinary shedding in vaccinated animals. As well as being nonpathogenic in domestic swine, 353-1 was found to not be shed from vaccinated animals. Both cell-mediated and humoral immune responses were found to be elicited by vaccination with 353-1. Although there are no established correlates of protection in the *Brucella* vaccinology field, proliferation of antigen stimulated peripheral blood mononuclear cells (PBMCs) has repeatedly been associated with vaccine efficacy in cattle and bison.\(^{16, 17}\) Proliferation was induced with antigen stimulation of PBMCs of 353-1 vaccinated animals. There were also significant differences in interferon gamma (IFN-\(\gamma\)) production by antigen stimulated PBMCs from vaccinated animals compared to controls. IFN-\(\gamma\) has been shown to be pivotal in the demonstration of a Th1 polarized cell-mediated immune response which is associated with clearance of intracellular pathogens in pigs.\(^{6, 7}\) The PBMC proliferation and IFN-\(\gamma\) responses were associated with reduced recovery of virulent *B. suis* from necropsy tissues four weeks after vaccinated pigs were challenged. Therefore, 353-1 possesses several characteristics of a favorable vaccine candidate: 1) It is nonpathogenic for the target species. 2) It is not shed into the environment from vaccinated animals. 3) After redistribution and systemic lymphoid infection, it is cleared from the body. 4) It is immunogenic. 5) It reduces colonization of virulent *B. suis* after challenge.

Although the initial trials with 353-1 are promising, there are still major hurdles to using a live vaccine in a feral swine population. *B. suis* is zoonotic, and is more virulent for humans than *B. abortus*. All work with 353-1 thus far has been conducted in a biosafety level 3 (BSL-3) environment. Further virulence trials are warranted before conducting work outside of a BSL-3 facility. Inoculation studies in mice and guinea pigs have been the hallmark of assessing virulence of *Brucella* strains,\(^{19, 22}\) and such studies are warranted with 353-1. Effects on nontarget species should also be determined. Because of these safety issues, live 353-1 may be a better vaccine candidate for domestic swine than feral swine. However, 353-1 is proof of the concept that swine can develop
protective immunity with *Brucella* vaccination and is a model for further investigations into *Brucella* infection and immunity in swine.

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