

Oral vaccination of sexually mature pigs with *Brucella abortus* vaccine strain RB51

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Objective—To develop a novel oral vaccine delivery system for swine, using the rough vaccine strain of *Brucella abortus*.

Animals—56 crossbred pigs from a brucellosis-free facility.

Procedure—In 3 separate experiments, pigs were orally vaccinated with doses of 1×10^9 to $> 1 \times 10^{11}$ CFU of strain RB51 vaccine. The vaccine was placed directly on the normal corn ration, placed inside a whole pecan, or mixed with cracked pecans and corn.

Results—Oral vaccination of pigs with vaccine strain RB51 resulted in a humoral immune response to strain RB51 and short-term colonization of the regional lymph nodes.

Conclusions and Clinical Relevance—A viscous liquid such as Karo corn syrup in association with pecans that scarify the oral mucosa are necessary when placing the live vaccine directly onto corn or other food rations. Doses of $> 1 \times 10^{11}$ CFU of RB51 organisms/pig in this mixture ensures 100% colonization of regional lymph nodes via the oral route. This method may allow an efficient and economical means to vaccinate feral swine for brucellosis. (*Am J Vet Res* 2001;62:1328–1331)

Brucella suis is a gram-negative facultative intracellular pathogen that causes the disease brucellosis in domestic and feral pigs.¹ Swine brucellosis is characterized by abortion, low production, infertility, and arthritis in infected pigs. *Brucella suis* can also cause a serious infection in human beings characterized by a systemic febrile illness. Veterinarians, farmers, research personnel, and abattoir employees are at risk for contracting this disease. Swine brucellosis is a recognized threat to domestic swine populations throughout the world.¹

The US government has been successful in eradicating *B suis* from domestic swine by eliminating

infected herds. However, a recognized obstacle to complete eradication of swine brucellosis is the large infected feral swine population known to be capable of transmitting *B suis* to domestic herds.² Because of the inherent problems in working with free-ranging wild pigs, individual test and removal seems impractical. Furthermore, because of the large numbers of infected pigs and limited monetary funds, such methods hold little promise in developing countries. A second possibility is vaccination by either direct inoculation of domestic pigs or oral delivery systems for feral populations. However, currently there is no acceptable vaccine for swine brucellosis in the United States.¹

A promising new candidate for swine is the vaccine *B abortus* strain RB51.³ Under experimental field conditions,⁴ strain RB51 protects sows from *B suis* infection. In that study, vaccinated sows were mated with infected boars whose semen yielded on average 10^4 to 10^5 CFU of *B suis* biovar 1/ml. The authors reported no difference in vaccine efficacy with varying doses (10^6 to 10^9 CFU), number of administrations (1 to 3), or inoculation route (IM or direct oral inoculation).

Results of studies in cattle indicate that calthood vaccination with strain RB51 provides protection equal or superior to the classic vaccine strain 19 (S19) following challenge with virulent *B abortus*.^{5,6} Strain RB51 is less pathogenic than S19 and more importantly does not result in serologic reactions that interfere with the traditional sero-diagnostic tests.^{3,5-7} Strain RB51 also provides cross-protection in vaccinated mice following challenge with virulent strains of *B suis*, *B melitensis*, or *B ovis*.⁸ Results of previous experiments by our laboratory and others indicate that cattle and mice can be successfully vaccinated orally with strain RB51.^{9,10} In the study presented here, we report on the development of an oral delivery system for strain RB51 in pigs that results in consistent colonization of regional lymph nodes and stimulation of the immune system.

Materials and Methods

Animals—Fifty-six crossbred pigs were purchased from the Louisiana State Agricultural Experiment Station swine research facility. Prior to vaccination, all pigs were seronegative for *Brucella*-specific antibodies on the basis of standard card testing and either RB51 specific ELISA or western immunoblot analysis.¹¹⁻¹³ Throughout the course of our study, all swine were housed in an approved restricted-access large animal isolation facility located at the Louisiana State University AgCenter, Baton Rouge, La. At the conclusion of the study, the pigs were euthanized at a government-inspected abattoir by use of the captive bolt and exsanguination method.

Vaccine—*Brucella abortus* strain RB51 was provided by the official US supplier of the vaccine.^a The vaccine was prepared and diluted according to the manufacturer's instructions,

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and viable counts were determined for each vial used. For vaccine doses higher than provided by the manufacturer, frozen stocks of *B abortus* strain RB51 were thawed and streaked on blood agar plates. Following incubation for 3 days at 37 C with 5% CO₂, bacteria were harvested, and vials containing > 1 × 10¹¹ CFU/ml were prepared as described previously.¹⁴

Experiment I—Twenty-four sexually mature nonpregnant gilts were placed into 12 groups consisting of 2 pigs each. Groups 1 through 6 were vaccinated SC in the right cervical area with 6 × 10⁹ or 6 × 10⁸ CFU of strain RB51, respectively (Table 1). Pigs from groups 5 and 6 received SC a booster dose of 1 × 10¹⁰ CFU of strain RB51 at 35 days after initial vaccination.

Groups 7 through 12 were vaccinated orally with a suspension containing either 2 × 10¹¹ or 2 × 10¹⁰ CFU of RB51, respectively. The oral inoculum consisted of the vaccine diluted in PBS solution and Karo^b corn syrup that was then poured over the usual ration. Each pig was individually observed to ensure that the full dose was consumed. The Karo corn syrup is believed to facilitate adherence of the vaccine organisms to the oral mucosa. Pigs from groups 11 and 12 were reexposed at 35 days after initial vaccination to an oral dose of 3.5 × 10¹⁰ CFU of RB51.

Following vaccination, groups of pigs that were vaccinated only once SC or orally were euthanatized and necropsied at 14, 28, or 42 days after primary immunization. Those pigs receiving 2 doses were necropsied at 42 days following the primary exposure (7 days following the booster dose).

Serum for serologic testing was collected prior to vaccination and weekly thereafter for analysis by use of a strain RB51 specific ELISA.¹³ On necropsy, tissue specimens were collected for bacteriologic culture from the suprapharyngeal, retropharyngeal, superficial cervical, portal, and internal iliac lymph nodes, and from the spleen, liver, uterus, and synovial fluid.

Experiment II—To evaluate another oral delivery method, 4 sexually mature nonpregnant gilts were vaccinated orally with pecans containing > 1 × 10¹¹ CFU of strain RB51 (Table 1). Each pecan was prepared by drilling a small hole in the nut and injecting the vaccine directly into the pecan. It was believed that the hard shell would scarify the oral mucosa, resulting in a more efficient exposure to the vaccine. Again, each pig was individually fed and observed to ensure complete ingestion of the nuts. At 7 days after feeding, the pigs were euthanatized, bled, and various specimens were obtained as described for experiment I.

Experiment III—Fifteen mature barrows and 13 nonpregnant sows were orally inoculated a number of times with varying doses of vaccine to mimic a field situation. The oral inoculum was prepared by mixing the vaccine diluted in PBS solution with an equal volume of Karo corn syrup and mixing the resulting slurry with whole pecans, pecan shells, and corn. Eighteen pigs were placed into 6 equal groups of 3 pigs each (groups 1 to 6). Each group received either 1 × 10¹⁰ or 1 × 10⁹ CFU of strain RB51 and was exposed 1, 2, or 3 times (Table 1). At 28 days after initial vaccination, the pigs were euthanatized, and specimens were obtained as described for experiment I. Sera were analyzed for strain RB51 specific antibodies by western immunoblot.¹² The remaining 10 pigs, groups 7 to 9, were inoculated 1 time with a high dose of > 1 × 10¹¹ CFU of strain RB51 via the same oral delivery system. The groups were necropsied at 14, 21, and 28 days after vaccination, respectively. At necropsy, tissue specimens were obtained for isolation of strain RB51 organisms.

Tissue preparation and bacteriologic culture—All tissue specimens were homogenized in sterile 0.9% NaCl solution and plated onto Farrell's selective medium¹⁵ containing 5% bovine blood; the limit of detection by use of this method is 13

Table 1—Experimental design and results of SC or oral inoculation of pigs with *Brucella abortus* vaccine strain RB51 in pigs

Group	Route	Vehicle	Initial Dose*	No. of pigs	Reexposure (route and dose)	Necropsy†	No. of serologic responders‡§	No. of pigs with positive culture results§
Experiment I								
1	SC	PBS	6 × 10 ⁹	2	ND	14	1	0
2	SC	PBS	6 × 10 ⁸	2	ND	14	1	0
3	SC	PBS	6 × 10 ⁹	2	ND	28	1	0
4	SC	PBS	6 × 10 ⁸	2	ND	28	1	1
5	SC	PBS	6 × 10 ⁹	2	SC, 1 × 10 ¹⁰	42	2	2
6	SC	PBS	6 × 10 ⁸	2	SC, 1 × 10 ¹⁰	42	2	0
7	Oral	Karo	2 × 10 ¹¹	2	ND	14	0	0
8	Oral	Karo	2 × 10 ¹⁰	2	ND	14	0	0
9	Oral	Karo	2 × 10 ¹¹	2	ND	28	0	0
10	Oral	Karo	2 × 10 ¹⁰	2	ND	28	0	0
11	Oral	Karo	2 × 10 ¹¹	2	Oral, 3.5 × 10 ¹⁰	42	0	0
12	Oral	Karo	2 × 10 ¹⁰	2	Oral, 3.5 × 10 ¹⁰	42	0	0
Experiment II								
1	Oral	Pecans	1 × 10 ¹²	4	ND	7	0	4
Experiment III								
1	Oral	PSK	1 × 10 ¹⁰	3	Oral, 2 doses, 1 × 10 ¹⁰	28	1	0
2	Oral	PSK	1 × 10 ¹⁰	3	Oral, 1 × 10 ¹⁰	28	2	0
3	Oral	PSK	1 × 10 ¹⁰	3	ND	28	3	0
4	Oral	PSK	1 × 10 ⁹	3	Oral, 2 doses, 1 × 10 ⁹	28	3	0
5	Oral	PSK	1 × 10 ⁹	3	Oral, 1 × 10 ⁹	28	3	0
6	Oral	PSK	1 × 10 ⁹	3	ND	28	3	0
7	Oral	PSK	1 × 10 ¹²	3	ND	14	0	3
8	Oral	PSK	1 × 10 ¹²	4	ND	21	2	4
9	Oral	PSK	1 × 10 ¹²	3	ND	28	3	3

*CFU/pig. †Days after primary vaccination. ‡Experiments I and II, RB51 specific ELISA, using whole killed strain RB51 as the antigen; Experiment III, Western immunoblot using strain RB51 cell lysate. §Number of pigs within a group.
 PBS = Phosphate buffered saline solution. ND = Not done. Karo = Karo corn syrup. PSK = Pecan shells mixed with Karo corn syrup.

CFU/g or ml. The plates were incubated for 14 days at 37 C in a 5% CO₂ atmosphere. Strain RB51 was identified by its ability to grow on rifampin-containing plates (250 µg/ml) and rough colony appearance.³ In experiments I and II, specimens from the liver, spleen, and uterus were individually homogenized and plated for each pig. In those pigs vaccinated SC, the superficial cervical lymph node was ground separately, because it directly drained the site of inoculation, whereas secondary lymph nodes, including the suprathypharyngeal, retrothypharyngeal, portal, and subiliac, were homogenized and plated together. The suprathypharyngeal and retrothypharyngeal lymph nodes from each orally vaccinated pig were ground and plated together as a primary lymph node, whereas the remaining lymph nodes were combined as a secondary lymph node. Synovial fluid was swabbed directly onto selective media. For experiment III, all of the lymph nodes were ground and plated as 1 homogenate, as were the liver and spleen. In experiment III, the uterus and synovial fluid were not submitted for bacteriologic culture of strain RB51 organisms.

Serologic analysis—Serum was analyzed for *Brucella* O-polysaccharide specific antibodies by the card test, standard tube agglutination, and western immunoblot analysis.^{11,12} In experiments I and II, RB51-specific serum antibodies were detected, using the strain RB51 antibody-specific ELISA that uses whole-killed strain RB51 organisms as the antigen.¹³ In experiment III, strain RB51 and *Brucella* O-polysaccharide specific serum antibodies were detected by western immunoblot analysis, using cell lysates from O-polysaccharide containing *B abortus* strain 2308 and O-polysaccharide-free strain RB51, as described previously.¹² Strain RB51 serologic reactions were recorded as either strong, moderate to weak, or negative on the basis of previously described procedures.¹⁶ Essentially, reaction strength was qualitatively determined on the basis of the number and intensity of strain RB51 proteins recognized by the test serum.

Results

Experiment I—Subcutaneous vaccination of 12 sexually mature nonpregnant gilts (groups 1 to 6) with *B abortus* strain RB51 did result in lymphoid colonization in 3 pigs and detectable serologic responses in 8 pigs (Table 1). On day 42, the organism was isolated after a booster dose of vaccine was administered in 2 pigs that initially received 6×10^9 CFU of strain RB51 SC but was not detected in 2 other pigs that received a booster dose of vaccine and had been vaccinated initially with 6×10^8 CFU SC. The vaccine strain was isolated from the superficial cervical lymph node of 1 of the pigs in group 5 and from a mixture of secondary lymph nodes from the other pig within that group. This mixture included the suprathypharyngeal, retrothypharyngeal, portal, and subiliac lymph nodes. Using strain RB51-specific ELISA, 4 of 8 pigs inoculated SC 1 time with strain RB51 (groups 1 to 4) developed detectable serum antibodies to strain RB51. One pig, although having positive culture results for strain RB51 organisms, was seronegative for strain RB51-specific antibodies. All pigs that received a booster dose of vaccine SC (groups 5 and 6) produced detectable anti-RB51 serum antibodies regardless of culture status.

Attempts to culture strain RB51 vaccine from the orally inoculated pigs (groups 7 to 12) were unsuccessful (Table 1). Also, no anti-RB51 serum antibodies were detected by use of the strain RB51-specific ELISA. Serum antibodies to the O-polysaccharide were not

detected in any of the test pigs by use of the card test or standard tube agglutination.

Experiment II—A second protocol for oral exposure was devised following the failure of the previous oral vaccination trial with strain RB51 in a Karo corn syrup vehicle to result in lymphoid colonization. To establish scarification of the oral mucosa and, hence, greater exposure to the vaccine, strain RB51 was placed inside whole pecans. This could be a practical delivery method, because feral pigs are known to readily consume pecans. Also, the oral inoculum was increased 100-fold to $> 1 \times 10^{11}$ CFU. Oral inoculation of 4 pigs with this method resulted in 100% colonization of the draining lymph node by day 7 after vaccination with CFU/g of tissue ranging from 1.61×10^2 to 7.46×10^2 . Strain RB51 was not cultured from the secondary lymph nodes, liver, spleen, uterus, or synovial fluid of these 4 pigs. Serum antibodies to strain RB51 were not detected by use of the strain RB51-specific ELISA, nor were anti-O-polysaccharide serum antibodies observed as the result of the card test and standard tube agglutination.

Experiment III—Following the successful lymphoid colonization of 4 pigs with strain RB51 inside a hard vehicle such as pecans, experiment III was initiated with another delivery system. Instead of the labor-intensive job of placing the vaccine inside whole pecans, the vaccine was mixed with Karo corn syrup and then laid on top of the normal corn feed along with whole and cracked pecan shells. In this experiment 3 different doses of either 1×10^9 , 1×10^{10} , or $> 1 \times 10^{11}$ CFU were used. Pigs were fed the mixture 1, 2, or 3 times (Table 1, experiment III, groups 1 to 6). On the basis of comparisons of western immunoblots,¹⁶ 15 of 18 pigs vaccinated with 1×10^{10} or 1×10^9 CFU of strain RB51 mounted a humoral immune response following vaccination. At necropsy, brucellae were not cultured from any of these pigs. In contrast, all of the pigs that received $> 1 \times 10^{11}$ CFU of the vaccine (groups 7 to 9) had tissue colonization at low numbers, on average approximately 100 CFU/g of tissue; 5 of 7 pigs developed serum antibodies to strain RB51 by day 21 after vaccination. Serum antibodies to the O-polysaccharide were not detected in any of the experimental pigs on the basis of the results of western immunoblot.

Discussion

As the United States attempts complete eradication of *B suis* from domestic swine herds, a growing concern is the threat infected feral pigs may have on domestic cattle and pigs.² The inherent difficulty in working with wild pigs makes direct vaccine inoculation impractical. Using a simple, cheap, yet efficient oral delivery method, it may be possible to vaccinate large numbers of feral hogs by pouring a vaccine directly onto attractive baits such as corn. Work with cattle and mice indicates that oral immunization with *B abortus* strain RB51 can lead to substantial protective immunity.^{9,10} Results of field studies performed in Venezuela⁴ indicate that direct disposition of strain RB51 on the upper surface of the pigs' tongues with a micropipette can provide protection from field strains. It is assumed

that for strain RB51 to generate a protective immune response, transient vaccine lymphoid colonization must occur in the lymph node draining the site of inoculation. On the basis of this assumption, our experiments were focused on identifying an inexpensive and practical vehicle to orally deliver strain RB51 and producing local lymphoid colonization.

In our study, we report the use of a corn and pecan mixture as a method of oral RB51 delivery resulting in local lymphoid colonization for at least 28 days. In experiment I, 12 gilts were vaccinated SC with strain RB51 to verify that the organism could colonize the draining lymph node. Vaccine organisms were isolated from primary and secondary lymph nodes from 3 pigs vaccinated SC (Table 1). However, no clear relationship exists between vaccine dose or frequency and isolation of vaccine organism. Eight of the pigs vaccinated SC developed strain RB51-specific serum antibodies on the basis of RB51-specific ELISA results, probably indicating transient lymphoid colonization. Subcutaneous inoculation of pigs with strain RB51 may produce a short-term infection not detectable by culture but sufficient to induce a detectable immune response and possibly protection.

All 12 pigs vaccinated orally in experiment I failed to produce strain RB51-specific serum antibodies as detected by ELISA and had negative bacteriologic culture results for strain RB51 organisms. In our opinion, a viscous vehicle such as Karo syrup when placed directly on the feed does not induce adequate scarification of the oral mucosa. Also, the test pigs may have ingested the normal ration so quickly that the vaccine did not come into sufficient contact with the nonscarified oral mucosa. Lord et al⁴ obtained good protection against infection by direct oral application of strain RB51 at doses ranging from 1×10^6 to 1×10^9 CFU. Because protective immunity was identified, it was assumed that colonization did occur during this field study.⁴ In our study, consistent lymphoid colonization with strain RB51 was not detected in pigs orally vaccinated with doses ranging from 1×10^9 to 1×10^{10} CFU. Only a dose of $> 1 \times 10^{11}$ CFU associated with mucosal membrane scarification led to reliable lymph node colonization (experiment III). Lord et al⁴ inadvertently induced mucosal and skin trauma by restraining pigs with an oral snare that may have allowed penetration, lymphoid colonization, and subsequent protection by strain RB51 even at doses below 1×10^{11} CFU.

The results of experiment II indicate that scarification along with a high vaccine dose can result in vaccine exposure and lymphoid colonization. In experiment II, it was found that the use of 1×10^{11} CFU of strain RB51 placed directly inside pecans resulted in colonization of the draining lymph node in all pigs by 7 days after vaccination. The pecans forced the pigs to crack each individual nut with their teeth, resulting in abrasions and longer contact between the mucosa and vaccine. Pigs were seronegative for strain RB51 antibodies, because only 7 days were allowed to elapse between exposure and necropsy.

Although direct inoculation of pigs by use of pecans worked well, the method is time consuming and requires experience. In an effort to find an easier

method, whole and cracked pecan shells were mixed with the strain RB51 organisms prior to feeding (experiment III). The results of this experiment indicate that variations in vaccine dose from 1×10^9 CFU to $> 1 \times 10^{11}$ CFU had an effect on lymphoid colonization but not necessarily on the humoral immune response. The mixture of strain RB51 with Karo corn syrup, corn, and pecan shells resulted in lymphoid colonization in all the pigs ingesting $> 1 \times 10^{11}$ organisms for at least 28 days after inoculation.

Oral vaccination of feral swine with strain RB51 should be possible by using a high vaccine dose in association with a viscous media such as Karo syrup and an oral scarification agent analogous to pecan shells. This mixture does not substantially alter the viability of the organisms, and the authors speculate that under field conditions the vaccine should be stable for > 48 hours.

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^bBest Foods Division, CPC International Inc, Englewood Cliffs, NJ.

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