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EFFICACY OF ORAL AND PARENTERAL ROUTES OF *MYCOBACTERIUM BOVIS* BACILLE CALMETTE-GUERIN VACCINATION AGAINST EXPERIMENTAL BOVINE TUBERCULOSIS IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*): A FEASIBILITY STUDY

P. Nol,[1,5,6] M. V. Palmer,[2] W. R. Waters,[2] F. E. Aldwell,[3] B. M. Buddle,[4] J. M. Triantis,[5] L. M. Linke,[5] G. E. Phillips,[1] T. C. Thacker,[2] J. C. Rhyan,[1] M. R. Dunbar,[1] and M. D. Salman[5]

[1] USDA/APHIS National Wildlife Research Center, Fort Collins, Colorado 80521, USA
[2] USDA/ARS National Animal Disease Center, Ames, Iowa 50010, USA
[3] Department of Microbiology and Immunology, University of Otago, P.O. Box 56, Dunedin, New Zealand
[4] AgResearch, Hopkirk Research Institute, Private Bag 1108, Palmerston North 4442, New Zealand
[5] Department of Clinical Sciences, Colorado State University, Fort Collins, Colorado 80523, USA
[6] Corresponding author (email: pauline.nol@aphis.usda.gov)

ABSTRACT: We investigated the efficacy of oral and parenteral *Mycobacterium bovis* bacille Calmette-Guerin Danish strain 1331 (BCG) in its ability to protect white-tailed deer (*Odocoileus virginianus*) against disease caused by *M. bovis* infection. Twenty-two white-tailed deer were divided into four groups. One group ($n=5$) received 10^9 colony-forming units (cfu) BCG via a lipid-formulated oral bait; one group ($n=5$) received 10^9 cfu BCG in culture directly to the oropharynx, one group ($n=6$) was vaccinated with 10^6 cfu BCG subcutaneously, and one group served as a control and received culture media directly to the oropharynx ($n=6$). All animals were challenged 3 mo after vaccination. Five months postchallenge the animals were examined for lesions. Results indicate that both oral forms of BCG and parenterally administerd BCG offered significant protection against *M. bovis* challenge as compared to controls. This study suggests that oral BCG vaccination may be a feasible means of controlling bovine tuberculosis in wild white-tailed deer populations.

Key words: BCG, bovine tuberculosis, *Mycobacterium bovis*, oral vaccination, vaccine, white-tailed deer.

INTRODUCTION

Bovine tuberculosis(BTb), caused by *Mycobacterium bovis,* poses a serious continual threat to the health and economic well-being of both livestock and humans worldwide. In the developing world, one third of the population lacks effective agricultural and food safety programs, leaving them at substantial risk for zoonotic infection by *M. bovis* (Nelson, 1999). In addition, wildlife reservoirs of BTb are believed to play a very important role in the epidemiology of this disease in many countries. In the United States, a free-ranging white-tailed deer (*Odocoileus virginianus*) population located in northeastern Michigan serves as such a reservoir (Schmitt et al., 1997; Fitzgerald et al., 2000; Palmer et al., 1999; 2000; Waters et al., 2004b).

In 1994 *M. bovis* was isolated from a hunter-killed white-tailed deer buck from northern Michigan. Subsequent surveillance of the free-ranging herd verified an epidemic presence of the disease. Several other wild mammalian species in the area have also been found to be infected or exposed to *M. bovis* including cervids and carnivores (Schmitt et al., 1997; Brunning-Fann et al., 2001). The self-sustaining presence of bovine tuberculosis in this wild deer population, and possibly other wild populations, poses a significant challenge to Michigan's efforts to reestablish its BTb-free status in domestic livestock. The implementation of an oral vaccination program, which would effectively reduce disease and shedding of *M. bovis* by deer and other affected species, would significantly aid efforts in eradicating BTb from Michigan.

We assessed the efficacy of orally administered *M. bovis* bacille Calmette-

Guerin Danish strain 1331 (BCG) in white-tailed deer against infection with virulent *M. bovis*. *Mycobacterium bovis* bacille Calmette-Guerin is currently the only vaccine licensed for use in humans against tuberculosis (*Mycobacterium tuberculosis*), and there are currently no accepted vaccination programs available to livestock. In fact, BCG remains the gold standard against which all experimental tuberculosis vaccines are compared, and despite intense efforts to develop a more effective vaccine, to date there are no new commercially available tuberculosis vaccines. Our study compared two types of oral BCG vaccination, as well as parenteral BCG vaccination, in their ability to protect white-tailed deer from disease caused by experimental infection of *M. bovis*.

MATERIALS AND METHODS

Animals

Thirty yearling female white-tailed deer were obtained from four BTb-free deer farms throughout the state of Iowa, USA, and were shipped to the USDA/ARS National Animal Disease Center (NADC) in Ames, Iowa, USA, in January 2005 and housed in an outdoor pen facility. Prior to shipment the animals were socialized as a group on one of the supplying farms for 3 wk. After delivery to NADC, animals were acclimated for 4 wk prior to start of the study. At time of challenge, deer were transferred to a Biosafety level (BL)-3 animal building. All deer were housed and cared for according to the Association for Assessment and Accreditation for Laboratory Animal Care International and institutional guidelines. This study was restricted to using a maximum of 30 animals due to space limitations.

Mycobacterium bovis BCG and *M. bovis* challenge strains

Mycobacterium bovis BCG Danish strain 1331 in culture and in lipid-formulated pellets were prepared at the University of Otago, Immune Solutions Ltd., as described in Aldwell et al. (2003a, b). Vaccine doses were determined using standard enumeration techniques by serial dilution plate counting on Middlebrook's 7H11 media (Becton-Dickinson, Cockysville, Maryland, USA). *Mycobacterium bovis* (strain 9839 Ames designation) was grown to midlog phase on Middlebrook 7H9 broth supplemented with 10% oleic acid-albumin-dextrose complex (OADC) (Difco, Detroit, Michigan, USA) plus 0.05% Tween 80. Bacilli were harvested from culture media by pelleting the cells by centrifugation at 2,000 × G, washing twice with 1 ml of phosphate-buffered saline solution and diluting to the appropriate cell density in 2 ml of PBS. The challenge dose was determined as described above for vaccine doses.

Vaccination and challenge of animals

White-tailed deer were orally vaccinated ($n=8$) with approximately 1×10^9 cfu BCG via lipid-formulated bait (oral bait group). These deer were each offered a 1 g, unflavored lipid pellet inside a piece of fresh apple and allowed to voluntarily eat the bait and apple so that they chewed the material before swallowing. Another group of eight deer were orally vaccinated with BCG in liquid medium (oral liquid group). A 1-ml preparation of approximately 1.9×10^8 cfu BCG in 7H9 broth was administered via 3-ml syringe and a 10 French 25-cm sterile urinary catheter (Self-Cath®, Mentor, Minneapolis, Minnesota, USA). Care was taken to contact the back of the mouth with the inoculum in order not to bypass the pharyngeal lymphoid tissue. Seven deer were vaccinated subcutaneously in the right caudal cervical area with a 1-ml preparation of approximately 3.4×10^6 cfu BCG (parenteral group). Seven deer received 1 ml 7H9 broth orally directly into the back of the mouth via syringe and catheter and served as the control group. Control and vaccinated deer were housed together in an outdoor paddock until challenge in an indoor BL3 facility.

At 12 wk post-vaccination, all animals were anesthetized with an intramuscular (IM) injection of ketamine hydrochloride (6 mg/kg) and xylazine (2 mg/kg), moved into a BL3 facility also at NADC, and immediately challenged with a total of 228 cfu *M. bovis* strain 9839 directly into the palatine tonsillar crypts (114 cfu/tonsil) via pipette, the dose being divided between the two tonsils. Oral liquid and parenteral doses of BCG and challenge dose of *M. bovis* were retrospectively determined via culture as described above. The oral bait dose, as estimated from culture of bacilli prior to lipid formulation, was approximately 9.2×10^8 cfu.

Mononuclear cell culture

Whole blood was collected by jugular venipuncture. Peripheral blood mononuclear cells (PBMCs) were obtained from whole

blood collected in 60-cc syringes containing 5 ml 2X acid citrate dextrose. PBMCs were isolated from buffy coat fractions using a Ficoll-Hypaque (Histopaque-1083, Sigma, St. Louis, Missouri, USA) technique followed by a lyse and restore step as described (Waters et al., 2004a). The PBMCs were resuspended in supplemented RPMI 1640 (Sigma) and counted. The RPMI 1640 was supplemented with 25 mM N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES) buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 50 μM 2-mercaptoethanol (Sigma), and 10% (v/v) fetal bovine sera (FBS; National Veterinary Services Laboratory [NVSL], Ames, Iowa, USA).

Lymphocyte blastogenesis

Lymphocyte blastogenesis assays were performed on days 0, 28, and 83 postvaccination. Mononuclear cells (5×10^5 cells) were added to wells of 96-well round-bottom microtiter plates (Falcon, Becton-Dickinson, Lincoln Park, New Jersey, USA). Wells contained medium plus 10 μg/ml *M. bovis* purified protein derivative (PPDb, Prionics AG, Schlieren, Switzerland), 10 μg/ml *Mycobacterium avium* PPD (PPDa, Prionics AG), 10 μg/ml rESAT6:CFP10 (E:C, Waters et al., 2004a), 1 μg/ml pokeweed mitogen (PWM, Sigma), or medium alone (no stimulation). Each antigen treatment was run in triplicate. Cells were incubated for 6 days at 37 C in 5% CO_2 in air, at which time each well was pulsed with 0.5 μCi methyl-[^{3}H]thymidine (Amersham Life Science, Arlington Heights, Illinois, USA) in 50 μl RPMI. Then PBMCs were incubated for another 24 hr and harvested onto fiber filters with a 96-well plate harvester (EG&G Wallace, Gaithersburg, Maryland, USA), and the radioactivity levels measured by liquid scintillation counting. Data are presented as stimulation indices (SI) where

$$\text{SI} = \frac{\text{Mean counts/min (antigen– or mitogen – stimulated cultures)}}{\text{Mean counts/min (nonstimulated cultures)}}.$$

Interferon-γ enzyme-linked immunosorbent assay

Interferon-γ (IFN- γ) responses to mycobacterial antigens were measured at −77, 0, 28, 49, and 83 days post-vaccination, and at 13, 52, 88, and 119 days post-challenge (98, 137, 173, and 204 days post-vaccination, respectively) using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cervigam™, Prionics AG) modified as described in Palmer et al. (2004b) to determine interferon production. Briefly, heparinized whole blood was collected by jugular venipuncture, of which 1.5 ml was added to 24-well tissue culture plates (Falcon, Becton-Dickinson, Lincoln Park, New Jersey, USA). Treatments administered included PBS (no stimulation), 20 μg/ml PPDb, 20 μg/ml PPDa, 20 μg/ml PWM, or 10 μg/ml E:C. Plates were incubated for 48 hr at 37 C in a humidified chamber with 5% CO_2. Samples were centrifuged at 400 × G, and the plasma supernatants harvested and stored at −80 C until analysis using the Cervigam™ ELISA according to the manufacturer's instructions. Optical density was measured at 450 nm using an automated ELISA microplate reader (Molecular Devices, Menlo Park, California, USA). Concentrations (ng/ml) of IFN-γ in test samples were determined by comparing absorbances of test samples with absorbances of standards within a linear curve fit (Instat 2.0, GraphPad Software Inc., San Diego, California, USA). The IFN-γ data (ng/ml) used for statistical analysis were derived from calculating the differences between response to antigen and response to no stimulation (PBS) (antigen stimulation (ng/ml)−no stimulation (ng/ml).

Post-mortem examination

Twenty weeks post-challenge, the deer were anesthetized with ketamine hydrochloride (6 mg/kg) and xylazine (2 mg/kg) and euthanized by intravenous administration of sodium pentobarbital (Sleepaway®, Fort Dodge, Iowa, USA). Tissues collected for gross pathology, histopathology, culture, and polymerase chain reaction (PCR) were palatine tonsils, mandibular lymph nodes (LNs), parotid LNs, medial retropharyngeal LNs, right prescapular LN, tracheobronchial LN, mediastinal LNs, right cranial lung lobe, liver, hepatic LN, and mesenteric LNs. In the case of bilateral LNs, excepting the prescapular LNs, portions of both right and left LNs were collected for both culture and histopathology. All lung lobes were carefully examined for lesions, and each was weighed individually for comparison with those of the other deer in the study. A scoring system based on number and extent of gross lesions was applied to all LNs collected and all lung lobes for statistical comparison (adapted from Vordermeier et al., 2002). For the lung lobes scoring was as follows: a score of "no lesions" meant there were no gross lesions detected. A score of "mild" represented tissues that contained any lesions fewer than or equal to five gross lesions of less than 10 mm in diameter. A score of "severe" was

assigned to tissues with greater than or equal to six gross lesions of less than 10 mm in diameter or any lesions larger than 10 mm in diameter. For the LNs scoring was as follows: a score of "no lesions" meant there were no gross lesions detected. A score of "mild" represented tissues that contained one small focus 1–2 mm in diameter. A score of "severe" was assigned to tissues with several small foci or extensive necrosis. All tissues for culture and PCR were stored in whirlpak bags at −80 C until testing. Tissues for histopathology were fixed in neutral-buffered 10% formalin and processed by routine paraffin embedment techniques. Sections were cut 5-μm thick, stained with hematoxylin and eosin, and examined under light microscopy. Sections containing microscopic lesions compatible with tuberculosis were subsequently stained with Ziehl-Neelsen for identification of acid-fast organisms. A scoring system was applied to all tissues collected for histopathology based on the number and extent of microscopic granulomatous lesions and was conducted as follows: a score of "no lesions" meant there were no granulomatous lesions detected; a score of "mild" represented tissues that had granulomas containing macrophages, giant cells, lymphocytes, and neutrophils, but had incomplete encapsulation and minimal to no necrosis present; a score of "severe" was given to any granulomas that displayed encapsulation, caseous necrosis, and mineralization.

Tissue culture and polymerase chain reaction

We selected right cranial lung lobe, mediastinal LN, and medial retropharyngeal LN for quantitative culture. Tonsil and mesenteric LNs were selected for general culture. In a Class 3 biosafety cabinet, a small piece of each tissue was removed and stored at −80 C for fresh tissue PCR analysis. All tissues were then homogenized using a blender (Oster, Shelton, Connecticut, USA) with 50 ml (LN) or 100 ml (lung) phenol red broth (NVSL). A 7.5-ml aliquot was removed and the remaining sample stored at −80 C for PCR analysis. The aliquot was then subjected to NaOH decontamination as follows. Five milliliters of 0.5 M NaOH was added to the 7.5-ml aliquot and allowed to incubate for 10 min. Then 10 N HCl was added dropwise until a yellow color was obtained followed by a dropwise addition of 1.0 N NaOH until a dusty rose color was achieved. Samples for general culture were centrifuged at 3,000 × G for 15 min. The supernatant was discarded, and the remaining sediment was used to inoculate three tubes of 7H10 media and three tubes of 7H11 media (NVSL). Samples for quantitative culture were vortexed at high speed for 5 sec and serially diluted at 1:10 dilutions in PBS out to 10^{-6}, vortexing for 5 sec between each dilution. One hundred microliters of each dilution was used to inoculate 7H11 agar plates (NVSL). The undiluted sample was used to inoculate one tube of 7H10 media. All cultures were placed in an incubator at 37 C. Plates were kept in plastic bags to prevent the agar from drying out. Samples were checked every 30 days, and final counts/assessments were made at 60 days.

Samples that produced growth on 7H10 and 7H11 media and considered suspect for *Mycobacterium* spp. were picked and stored in 1 ml phosphate buffered saline at −80 C for follow-up PCR to confirm or rule out the presence of *M. bovis.* Extraction of DNA from cell culture samples was performed using the BioRad InstaGene™matrix (Bio-Rad Laboratories, Hercules, California, USA) according to the manufacturer's instructions with the following modifications: Briefly, each sample was vortexed, after which 200 μl of cell homogenized sample was transferred into a bead beater tube (Sarstedt Inc., Newton, North Carolina, USA) containing 0.5 g of 0.5-mm zirconium/silica beads (Biospec Products Inc., Bartlesville, Oklahoma, USA). Samples were bead beated using the MBB-8 Mini Bead Beater (Biospec Products) for 2 cycles (4,500 rpm) of 10 sec. After a quick spin, 50 μl of bead-beated cells were transferred to a new tube, to which 200 μl of Instagene Chelex matrix was added. Next, 14.2 μl of proteinase K (Amresco Inc., Solon, Ohio, USA; 20 mg/ml) was added to each sample, which, after vortexing, was then incubated at 60 C for 30 min. The proteinase K was inactivated by heating the samples to 100 C for 10 min. Finally, the samples were centrifuged at 11,000 × G for 10 min, and the resulting supernatants containing DNA were saved. For the purposes of PCR, dilutions were made of the DNA samples ranging from 1:10 to 1:10,000 depending on extent of turbidity. For every extraction, one tube containing 200 μl of PBS was included halfway between the total sample size and at the end of the extraction samples to serve as appropriate negative extraction controls.

The samples were then analyzed for *M. tuberculosis* complex DNA using a PCR targeting the IS6110 regions of *M. tuberculosis* complex (IS6110 PCR). The IS6110 PCR was performed using a PTC-100 MJ Thermalycler (MJ Research Inc., Waltham, Massachusetts, USA) for 50 cycles. The primers used in the IS6110 PCR were as follows: forward primer

(5′CTCGTCCAGCGCCGCTTCGG3′) and reverse primer (3′CCTGCGAGCGTAGGC GTCGG5′) (Operon Biotechnologies, Inc., Huntsville, Alabama, USA). Each IS6110PCR reaction (25 μL) occurred in a 0.65 μL PCR tube (Sorenson BioScience Inc., Salt Lake City, Utah, USA) and consisted of 1X GeneAmp Buffer II, 1.5 mM MgCl (Applied Biosystems, Foster City, California, USA), 2.5 mM of each deoxynucleoside triphosphate (Roche Applied Science, Indianapolis, Indiana, USA), 0.4 μM of each primer, 1.875 units of AmpliTaq Gold Polymerase (Applied Biosystems), 10 μL template, and 6.625 μL PCR-grade water (Hyclone, Logan, Utah, USA) covered with a 30 μL layer of Chill-Out wax buffer (Bio-Rad Laboratories). An amplification profile of 1) 94 C for 10 min, 2) 94 C for 45 sec, 3) slope −22 degrees at 1 degree C/1 sec, 4) 72 C for 2.25 min, 5) slope +22 degrees at 1.5 degrees C/1 sec, 6) repeat 2–5 for 49 cycles, and 7) 72 C for 2.25 min was followed by a final extension of 72 C for 10 min.

A positive control (purified *M. bovis* DNA at 0.5–5 fg/μL) and a negative control (water) were included in every experiment. Following the amplification protocol, 10 μl of the amplification reactions were size fractionated through 2.5% agarose (Amresco, Inc.) gels in 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA) (Amresco, Inc.). Gels were stained in 0.001 mg/ml ethidium bromide (Amresco, Inc.) for 15 min, and products were then visualized using the BioRad Gel Doc EQ System (Bio-Rad Laboratories) UV transilluminator. Product size was 322 base pairs.

Medial retropharyngeal LN, mediastinal LN, and right cranial lung lobe–derived fresh and homogenized tissues were also directly tested for *M. tuberculosis* complex by PCR. Frozen homogenates were placed at 4 C overnight to thaw. In a class 2 biosafety cabinet, approximately 1.3 ml homogenate was removed from the bottom of the 50-ml polypropylene centrifuge tube and transferred to a 2-ml polypropylene centrifuge tube. Samples were centrifuged for 10 min at 11,000 × G. Saving the resultant phenol broth supernatant in a new tube, 100 mg of the tissue was weighed out and placed into a 2-ml bead beater tube containing 2.5 g of 2.5 mm and 0.5 g of 0.5 mm zirconia/silica beads, to which 100 μl of the supernatant was returned. For every nine samples, a bead beater tube was filled with 200 μl PBS to serve as negative control. All tubes were quick spun and placed in a heat block for 10 min at 100 C. The samples were allowed to cool for 10 min. Tubes were then bead beated for three cycles (4,500 rpm) of 30 sec. Samples were cooled in an ice block in between cycles to avoid overheating. At this point samples were considered safe to handle outside the biosafety cabinet. DNA extraction was then accomplished using the Fermentas Genomic DNA Purification Kit® according to manufacturer's instructions (Fermentas Life Sciences, Hanover, Maryland, USA). Following the manufacturer's recommended DNA ethanol (ETOH)/salt precipitation, one Breath-Easier® tube membrane (ISC Bioexpress, Kaysville, Utah, USA) was placed over each open tube to avoid cross-contamination and allow for proper ETOH evaporation while incubating at 65 C under a still-air hood. DNA samples of the homogenized tissue were rehydrated in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA) (Amresco Inc.) and diluted to 1:60 and 1:80 in PCR-grade water for PCR.

In a class 2 biosafety cabinet, 100 mg of fresh frozen tissue was weighed out from each sample for DNA extraction. Tissues were minced and placed in bead beater tubes, containing 2.5 g of 2.5 mm and 0.5 g of 0.5 mm zirconia/silica beads, to which 100 μl of TE buffer was added. Tissues underwent bead beating homogenization with two pulses of 30 sec at 4,500 rpm. Samples were cooled in an ice block in between cycles to avoid overheating. The Epicentre MasterPure™ DNA Purification Kit was used according to the manufacturer's instructions with some modifications (Epicentre Biotechnologies, Madison, Wisconsin, USA). Briefly, samples were quick spun, and 500 μl of T and C Lysis Solution with 0.675 mg/ml proteinase K was added to each sample. Samples were incubated for 60 min at 56 C and again for 10 min at 100 C to inactivate the proteinase K and render any remaining *M. bovis* nonviable. After the samples cooled, 250 μl of MPC Protein Precipitation Reagent was added, and samples were centrifuged at 11,000 × G for 10 min at 4 C. The supernatant was then treated with 750 μl of 100% isopropanol and centrifuged for 15 min at 11,000 × G at 4 C. The isopropanol was discarded, and the pellet was washed two times with 75% ETOH. Any remaining ETOH was allowed to evaporate by placing a Breath-Easier® tube membrane over each open tube as described above. DNA samples of the fresh tissue were rehydrated in 100 μl of TE buffer and diluted to 1:60 and 1:80 for IS6110 PCR.

DNA from fresh and homogenized tissues underwent PCR analysis as described above. Any DNA samples deemed positive were subjected to two repeat PCR analyses, and

corresponding tissue samples were subjected to one additional extraction and PCR analysis to confirm presence of *M. bovis*. Unlike culture, these assays do not reflect the viability of the mycobacteria, but serve as a sensitive method to test for evidence of infection at some time during the experiment.

Statistical analysis

Fisher's Exact Test (4×3; one-sided) was used to compare all groups based on number of animals with gross lesions (none, mild, and severe) and microscopic lesions (none, mild, and severe) (Samuels and Witmer, 1999) (Proc FREQ; SAS 9.1, SAS Institute, Cary, North Carolina, USA). If an association existed, Fisher's Exact Test (2×2; one-sided) was again used to compare the number of animals with lesions within each vaccine group to the number of animals with lesions within the control group or to each other based on no lesions versus lesions (mild and severe combined). Culture and PCR results were compared in the same manner (Fisher's Exact Test; 2×2; one-sided) in order to determine differences in number of culture/PCR positive animals among groups. As this was a resource-intensive pilot study that was limited to a small number of animals, statistical priority was placed on minimizing the chance of type 2 error in order to detect differences if they were biologically valid. It was determined that using 8 animals per group, at an alpha level of 0.1, the test would have a power of 0.8 in detecting a difference when the proportion of animals without lesions in the vaccine groups exceeded the proportion of animals without lesions in the control group by 0.5 (SAS Institute, 2006). Therefore, differences determined by the one-sided Fisher's Exact Test to have $P \leq 0.1$ were considered significant.

We modeled IFN-γ and lymphocyte blastogenesis response variables as functions of treatment group, time point, and group×time point interaction. We coupled this fixed-effects model structure with three alternative covariance structures to account for random effects of individual deer, effects of repeated measures within individuals, and unequal variances among treatment groups using the GLIMMIX procedure of SAS (Littell et al., 2006; SAS Institute 2006). The most general covariance structure estimated separate variance components and autoregressive correlation coefficients by treatment group. Models with reduced covariance structures included one model estimating a common variance component (random effect of individuals) and a common autoregressive correlation among treatment groups, and one model estimating a common variance component (i.e., no treatment-group covariance effect, no repeated measures effect). We used an information theoretic approach to select the most parsimonious form of covariance structure for each response variable (Burnham and Anderson, 2002; Littell et al., 2006). For the selected models, we considered fixed effects as significant for $P \leq 0.1$.

RESULTS

Animals

Because of illnesses and injuries, eight animals were removed at various times throughout the study. Twenty-two animals remained in the study at the time of post-mortem examination. Of these 22 animals, five were in the oral bait group, five were in the oral liquid group, six were in the parenteral group, and six were in the control group.

IFN-γ and lymphocyte proliferative responses to mycobacterial antigens

Overall there was no detectable interaction between group and sampling time regarding IFN-γ responses to PPDb or E:C ($P=0.25$ and 0.39, respectively; data not shown). There was a significant interaction detected regarding IFN-γ responses to PPDa, although this interaction is based on responses obtained at only one time point (52 days post-challenge; 137 days post-vaccination; data not shown). Least squares means estimates of vaccinates were compared to those of the control group as well. At 28 days post-vaccination and at 13 days post-challenge (98 days post-vaccination) the parenteral group produced significantly greater levels of response to PPDb relative to the control group ($P=0.09$ and $P=0.057$, respectively). At 52 days post-challenge (137 days post-vaccination) the parenteral group exhibited increased reactivity to PPDa in relation to the control group ($P=0.062$), whereas the oral group exhibited decreased reactivity to PPDa ($P=0.0025$). The control group showed greater reactivity to PPDb and E:C than

TABLE 1. Microscopic lesions in the right cranial lung lobe and mediastinal lymph nodes of deer vaccinated with *M. bovis* BCG Danish strain 1331 via oral and parenteral routes versus deer vaccinated with medium alone.[a]

Tissue[b]		Vaccine group			
		Oral liquid	Oral bait	Parenteral	Control[c]
MLNs	Culture pos/total	0/5	0/5	0/6	4/6*
	(Mean cfu/g)[d]	NA	NA	NA	(5.08±1.6) (n=3)
	PCR pos/total	0/4	0/5	0/6	4/6*
	Lesions[e] pos/total	0/5	0/5	3/5†	6/6†
RCL	Culture pos/total	0/5	0/5	0/6	3/6
	(Mean cfu/g)	NA	NA	NA	(3.49±0.16) (n=3)
	PCR pos/total	0/4	0/5	1/6	6/6*
	Lesions pos/total	0/5	0/5	1/6	6/6*
MRLN	Culture pos/total	2/5	0/5	1/6	1/6
	(Mean cfu/g)	2/4	NA	(3.4)	(4.61)
	PCR pos/total	2/4	0/5	1/6	2/6
	Lesions pos/total	2/5	0/5	1/6	1/6

[a] All deer were challenged with *M. bovis* strain 9839 3 mo after vaccination. Tissues were collected 5 mo after challenge. For the right cranial lung lobe, scoring was as follows: None = no gross lesions detected; Mild = five or fewer gross lesions of less than 10 mm in diameter; Severe = six or more gross lesions of less than 10 mm in diameter or any lesions larger than 10 mm in diameter. For the mediastinal lymph nodes scoring was as follows: None = no gross lesions detected; Mild = small focus 1–2 mm in diameter; Severe = several small foci greater or equal to 5 mm or extensive necrosis.

[b] MLNs = mediastinal lymph nodes; RCL = right cranial lung lobe; MRLN = medial retropharyngeal lymph node.

[c] *Significantly different from all three vaccine groups (Fisher's Exact Test; $P \leq 0.1$); †significantly different from Oral Liquid and Oral Bait groups (Fisher's Exact Test; $P < 0.1$).

[d] Mean bacterial count reported as $\log_{10}$ cfu/g ±SD.

[e] Microscopic lesions

all three vaccine groups at 52 days post-challenge ($P < 0.096$) (data not shown).

There were no detectable differences in lymphocyte blastogenic responses to any of the *M. bovis* antigens after vaccination between vaccine groups and the control group individually or over time ($P \geq 0.57$; data not shown).

Post-mortem examination

When present, gross or microscopic lesions consistent with tuberculous lesions were found in the right cranial lung lobe and mediastinal LNs. Lesions were not consistently observed in any other tissues, including medial retropharyngeal LNs, although they were present. Therefore, only the right cranial lung lobe and mediastinal LN were suitable for statistical analysis.

The number of animals sustaining gross lesions in the right cranial lung lobe and mediastinal LNs in the vaccine groups was significantly lower than in the control group (oral bait—0/5; oral liquid—1/5; parenteral—1/6; control—6/6 for cranial lung lobe; oral bait—0/5; oral liquid—0/5; parenteral—1/6; control—6/6 for mediastinal LN; $P < 0.02$) (Table 1). No differences could be detected among the vaccine groups regarding the number of lesions in either right cranial lung lobe or mediastinal LN ($P = 1.0$).

The numbers of animals with microscopic lesions observed in the right cranial lung lobe and mediastinal LN of the oral liquid and oral bait groups were significantly lower than in the control group (oral bait—0/5, oral liquid—0/5, control—6/6 for cranial lung lobe; oral bait—0/5, oral liquid—0/5, control—6/6 for mediastinal LN; $P = 0.002$). In the parenteral group, there were fewer animals having histologic lesions in the right cranial lung lobe compared to the control group (parenteral—1/6, control—6/6; $P = 0.03$).

TABLE 2. Number of deer that are positive for culture, PCR, or microscopic lesions in selected tissues over total number of animals tested in the treatment group.[a]

	Right cranial lung lobe			Mediastinal lymph nodes		
Vaccine group	None	Mild	Severe	None	Mild	Severe
Oral liquid (n=5)	5	0	0	5	0	0
Oral bait (n=5)	4	1	0	5	0	0
Parenteral (n=6)	5	0	1	5	1	0
Control (n=6)	0	0	6[b]	0	0	6[b]

[a] All deer were challenged with *M. bovis* strain 9839, 3 mo after vaccination. Tissues were collected 5 mo after challenge. None = no granulomatous lesions detected; Mild = presence of granulomas containing macrophages, giant cells, lymphocytes, and neutrophils, but having incomplete encapsulation and minimal to no necrosis present; Severe = presence of granulomas displaying encapsulation, caseous necrosis, and mineralization.

[b] Significantly different from all three vaccine groups (Fisher's Exact Test; $P<0.1$).

However, regarding the mediastinal LN, the parenteral group did not appear to differ from the control group (parenteral—3/5, control—6/6; $P=0.18$), but did differ from both oral groups, having more animals with microscopic lesions in that tissue compared to the other vaccine groups ($P=0.08$; Tables 2 and 3). No differences could be detected among the vaccine groups regarding the number of microscopic lesions in the right cranial lung lobe ($P=0.13$). Microscopic lesions in the medial retropharyngeal LNs were found in two animals from the oral liquid group, one animal from the parenteral group, and one animal from the control group. Again, these data were not subjected to statistical analysis and are summarized in Table 3.

Presence of *M. tuberculosis* complex in tissues

When looking at all five tissues cultured (right cranial lung, mediastinal LNs, medial retropharyngeal LNs, tonsil, mesenteric LNs), the oral bait and parenteral groups, but not the oral liquid group, had significantly fewer culture positive animals than did the control group in which all six animals were positive (oral bait—0/5 pos., $P=0.002$; parenteral—2/6 pos., $P=0.03$; oral liquid—3/5 pos., $P=0.18$). No significant differences were noted among the vaccine groups ($P=0.18$). When looking at differences in individual tissues (Table 3), there were fewer animals in the oral liquid, oral bait, and parenteral groups that were culture positive for *M. tuberculosis* complex in the mediastinal LNs than in the control group (oral liquid—0/5, oral

TABLE 3. Gross lesions in the right cranial lung lobe and mediastinal lymph nodes of deer vaccinated with *M. bovis* BCG Danish strain 1331 via oral and parenteral routes versus deer vaccinated with medium alone.[a]

	Right cranial lung lobe			Mediastinal lymph nodes		
Vaccine group	None	Mild	Severe	None	Mild	Severe
Oral liquid (n=5)	5	0	0	5	0	0
Oral bait (n=5)	5	0	0	5	0	0
Parenteral (n=6)	5	0	1	2	1	2[b]
Control (n=6)	0	0	6[c]	0	0	6[b]

[a] Deer were vaccinated with *M. bovis* BCG Danish strain 1331 via oral and parenteral routes or received medium alone. All deer were challenged with *M. bovis* strain 9839, 3 mo after vaccination. Tissues were collected 5 mo after challenge.

[b] Significantly different from Oral Liquid and Oral Bait groups (Fisher's Exact Test; $P<0.1$).

[c] Significantly different from all three vaccine groups (Fisher's Exact Test; $P\leq0.1$).

bait—0/5, parenteral—0/6, control—4/6; $P \leq 0.045$). Regarding the right cranial lung, there were differences, although not significant differences, detected between the vaccine groups and the control group (oral liquid—0/5, oral bait—0/5, parenteral—0/6, control—3/6; $P = 0.18$). No differences were observed among the vaccine groups either. No differences were seen in the medial retropharyngeal LN cultures (Table 3). Quantitative culture results supported the above findings that *M. bovis* was more readily cultured out of mediastinal LNs and right cranial lung lobe collected from unvaccinated animals than in tissues from vaccinated animals. These results are reported in Table 3; however, they were not subjected to statistical analysis due to small sample size.

When PCR was applied directly to tissues and tissue homogenates, we observed similar trends but greater differences among the groups as compared to culture regarding presence of *M. tuberculosis* complex in right cranial lung lobe and mediastinal LNs (Table 3). The number of animals testing PCR positive for *M. tuberculosis* complex in mediastinal LNs in the three vaccine groups were significantly fewer than in the control group (oral liquid—0/4, oral bait—0/5, parenteral—0/6, control—4/6; $P \leq 0.008$). As well, there were significantly fewer animals testing PCR positive in the right cranial lung lobe in the three vaccine groups than in the control group (oral liquid—0/4, oral bait—0/5, parenteral—1/6, control—6/6; $P \leq 0.072$). Again there were no differences noted among the vaccine groups ($P = 0.28$). Culture, PCR, and histopathologic results in the three tissues are summarized in Table 3.

DISCUSSION

The present study indicates that oral BCG Danish 1331 is effective in protecting white-tailed deer against disease caused by experimental *M. bovis* infection. Orally vaccinated deer had fewer tuberculous lesions, both gross and microscopic, than did control deer 5 mo after challenge. To the authors' knowledge, this is the first study reporting efficacy of oral BCG in this species in experimental settings. Waters et al. (2004b) evaluated cellular immune responses in white-tailed deer vaccinated subcutaneously with BCG Pasteur, and demonstrated strong IFN-γ and proliferative responses to PPDb, suggesting that BCG may be protective against *M. bovis* infection in this species. Challenge with *M. bovis* was not performed in this study. Miller and others (1999) orally administered a recombinant BCG expressing *Borrelia burgdorferi* Osp A to white-tailed deer and detected both cellular and humoral immune response to *M. bovis* antigen, evidence that BCG was taken up via oral route. However, potential efficacy of the vaccine against *M. bovis* infection was not investigated, as the sole purpose of this study was to test the feasibility of BCG as an oral delivery system for nonmycobacterial antigens. In a New Zealand study investigating BCG (Pasteur 1173P2) in red deer (*Cervus elaphus*), researchers vaccinated animals directly in the tonsilar crypt (Griffin et al., 1999). Similar to oral BCG in white-tailed deer, the New Zealand scientists demonstrated that one dose of 2.5×10^6 cfu BCG into the tonsilar crypt of red deer protected 8 of 10 animals from disease 21 wk after *M. bovis* challenge, and 5 of 10 animals were culture negative. Red deer that were vaccinated subcutaneously with the same single dose of BCG experienced comparable protection, also similar to the white-tailed deer in this study. It should be noted that this and subsequent studies also demonstrated that red deer vaccinated with two subcutaneous doses of BCG 4–8 wk apart fared better than either subcutaneous or intratonsilar single-dose groups after challenge (Griffin et al., 2006).

The results of the present experiment indicate that white-tailed deer can be

vaccinated orally using BCG incorporated in a lipid-formulated bait. This oral bait has successfully induced protection against *M. bovis* and *M. tuberculosis* infection in a number of species, including laboratory mice (Aldwell et al., 2003), brushtail possum (*Trichosurus vulpecula;* Aldwell et al., 2003a), European badger (*Meles meles;* S. Lesellier, pers. comm.), and domestic cattle (Buddle et al., 2005). Buddle and others (2005) first demonstrated that ruminants can be vaccinated using this method. In their study, calves fed 10^9 cfu BCG (Pasteur 1173P2) via oral bait and challenged 21 wk after vaccination had significantly lower lung and LN lesion scores than did nonvaccinated cattle. The current findings are consistent with this previous study and suggest that white-tailed deer are a good candidate for oral vaccination programs to control bovine tuberculosis in the field.

This study examined the level of protection provided by oral BCG in white-tailed deer 5 mo post-challenge. Although the level of protection was significant based on analyses of lung and mediastinal LNs, these data do not suggest that a lack of lesions equates to sterile immunity. A few animals still had evidence of infection, based on histologic lesions or positive detection of *M. bovis* in medial retropharyngeal LNs and occasionally in other tissues as well (data not shown). For this reason it was important to test tissues in parallel via histopathology, PCR, and culture, as culture for Mycobacteria is quite difficult and is therefore relatively insensitive as an independent test. It would be of great interest to evaluate disease progression, if any, in this species in subsequent vaccine trials through extending the time between challenge and postmortem examination. Griffin and others (2006) addressed this question in red deer and found that animals vaccinated subcutaneously 8 wk apart remained refractory to disease up to 52 wk, despite the fact that they harbored low numbers of *M. bovis* in their tissues. Animals with stable, nonprogressive lesions are less likely to play an active role in disease transmission (Griffin et al., 2006). Therefore, vaccines that do not produce universal sterile immunity but do significantly limit disease still have great potential to control endemic tuberculosis in wildlife.

The predominant sites of mucosal uptake of oral BCG in ruminants are currently unknown. It has been shown in rabbits that mycobacteria can cross the mucosal barrier through M cells present in gut-associated lymphoid tissues, namely, Peyer's patches (Fujimura, 1986; Tizzard, 2004). In Lagranderie et al. (2000), mice vaccinated orally with 2×10^9 cfu BCG transiently harbored BCG in both the submaxillary glands and Peyer's patches immediately post-vaccination. However, only the periglandular LNs in the submaxillary region maintained high numbers of bacilli 84 days after administration of BCG when compared to the mesenteric LNs, suggesting that primary uptake occurs in the pharyngeal region, where nasopharyngeal lymphoid tissues are present. In contrast, mice which received the same dose intragastrically maintained higher numbers of bacilli in the mesenteric LNs. Aldwell and others (2005a, 2006) inoculated mice with 5.1×10^7 cfu lipid-microencapsulated BCG and found that the major site of bacterial replication was the mesenteric LNs, indicating that the lipid encapsulation reduced uptake in the pharyngeal region and protected the attenuated bacillus from destruction in the stomach. However, Dorer et al. (2007) showed BCG to be present in cervical and mesenteric LNs and Peyer's patches of mice 8 wk after receiving $1–2\times10^7$ cfu lipid-encapsulated BCG. Similar studies in other species corroborate these findings, although they all utilize a monogastric model. It is therefore of vital importance to investigate the potential impact of the complex ruminant gastrointestinal system upon both protected and nonprotected BCG in future studies.

Increases in IFN-γ production and proliferation by lymphocytes in the presence of *M. bovis* antigens can be used as indicators that an appropriate cellular immune response to BCG vaccination has occurred. In infected deer, however, robust IFN-γ responses can imply the presence of more severe disease. Thacker et al. (2006) found that PBMC from white-tailed deer with severe pathology due to *M. bovis* infection had greater IFN-γ mRNA expression to PPDb and E:C early on in the infection than did animals with pathology limited to the head LNs. As infection progressed the IFN-γ responses of the low-pathology group increased to those of the high-pathology group. Although the deer in this study responded well to vaccination, we could not have predicted this based on our IFN-γ or lymphoproliferative data prior to challenge. We also did not observe important differences in IFN-γ production between controls and vaccinates after infection. It is interesting that the group that received subcutaneous BCG vaccination displayed a degree of vacillation in IFN-γ responses not seen in any of the other groups over the course of the study. Although this may be due to the difference in the character of the immune response to BCG and *M. bovis* by this group, there may be other factors involved, such as the influence of exposure to antigens of *M. avium.* An important aspect to consider is that, at multiple time points, samples from up to 20% of the animals did not show an IFN-γ response to any antigen, based on the Cervigam ELISA, including the pokeweed mitogen control. Similar results were reported in a study by Waters et al. (in press) evaluating Cervigam as a diagnostic tool in various deer species, wherein only 44% of white-tailed deer had responses to PWM, calling into question the validity of this test for this species. In addition, the IFN-γ and lymphocyte responses observed in individual deer within groups were extremely variable. It is quite likely that, had our sample sizes been larger, we would have obtained data more consistent with those reported in other studies.

Experiments involving large, highly stressed species under BL-3 conditions are typically limited by space and cost. Power and significance levels need to be carefully considered prior to carrying out such a study and analyzing the results. Vaccine studies with low sample sizes are particularly susceptible to producing outcomes that apparently fail to detect differences between or among treatment groups when there actually is a treatment effect. It is therefore justified to increase the alpha level and hence choose a higher *P* value in order to increase the power of the test and avoid type 2 error that renders otherwise valuable data meaningless in a practical sense. The purpose of this pilot study was to investigate the feasibility of pursuing further research with oral BCG in white-tailed deer. Despite the fact that our numbers were very small, we were still able to clearly show that BCG does appear to be effective in protecting deer from disease.

In conclusion, the use of BCG for oral vaccination of white-tailed deer shows great potential in controlling disease caused by *M. bovis* infection. Oral vaccination significantly slowed pathologic progression of disease in our animals over a 5-mo period and thus is a promising candidate for use in field. Oral vaccination of wildlife is a management tool that has been successfully implemented in Europe to control rabies in red fox (*Vulpes vulpes;* Brochier et al., 1989) and is currently being used in the United States to manage the disease in raccoons (*Procyon lotor*). Oral BCG vaccination of wildlife reservoirs of bovine tuberculosis is being extensively researched for application in many countries, including New Zealand, United Kingdom, Ireland, United States, and South Africa. Successful mucosal delivery (intranasal or intraconjunctival) of BCG to a wild population of brushtail possums in New Zealand has already been

demonstrated by Corner et al. (2002). The results of this study provide strong support for further research that could ultimately lead to safe and successful field vaccination of white-tailed deer and eventual eradication of bovine tuberculosis in the United States.

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