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Vaccination with *Mycobacterium bovis* BCG Strains Danish and Pasteur in White-tailed Deer (*Odocoileus virginianus*) Experimentally Challenged with *Mycobacterium bovis*

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ORIGINAL ARTICLE

Vaccination with *Mycobacterium bovis* BCG Strains Danish and Pasteur in White-tailed Deer (*Odocoileus virginianus*) Experimentally Challenged with *Mycobacterium bovis*

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Impacts

- Bovine tuberculosis is an important zoonotic disease, for which most industrialized nations have eradication programmes.
- A serious obstacle to many eradication programmes worldwide is the presence of wildlife reservoirs of *Mycobacterium bovis*. Vaccination is one tool that may mitigate transmission from wildlife to domestic animals.
- Vaccination of white-tailed deer with *M. bovis* BCG Danish or Pasteur decreases disease severity; however, vaccine persists within tissues for as long as 250 days, can induce lesions in regional lymph nodes and can be transmitted between vaccinated and non-vaccinated deer.

Keywords:
BCG; Danish; deer; *Mycobacterium bovis;* Pasteur; tuberculosis; vaccination

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Summary

Wildlife reservoirs of *Mycobacterium bovis* represent serious obstacles to the eradication of tuberculosis in domestic livestock and the cause for many faltering bovine tuberculosis eradication programmes. One approach in dealing with wildlife reservoirs of disease is to interrupt inter-species and intraspecies transmission through vaccination of deer or cattle. To evaluate the efficacy of BCG vaccination in white-tailed deer, 35 deer were assigned to one of three groups; one s.c. dose of 10^7 CFU of *M. bovis* BCG Pasteur ($n = 12$); 1 s.c. dose of 10^7 CFU of *M. bovis* BCG Danish ($n = 11$); or unvaccinated deer ($n = 12$). After vaccination, deer were inoculated intratonsilarly with virulent *M. bovis*. Lesion severity scores of the medial retropharyngeal lymph node, as well as all lymph nodes combined, were reduced in vaccinated deer compared to unvaccinated deer. BCG Danish vaccinated deer had no late stage granulomas characterized by coalescent caseonecrotic granulomas containing numerous acid-fast bacilli compared to BCG Pasteur vaccinated or unvaccinated deer where such lesions were present. Both BCG strains were isolated as late as 250 days after vaccination from deer that were vaccinated but not challenged. In white-tailed deer, BCG provides protection against challenge with virulent *M. bovis*. Issues related to vaccine persistence, safety and shedding remain to be further investigated.

Introduction

Mycobacterium bovis is the causative agent of tuberculosis in animals and has one of the broadest host ranges of any pathogen. *Mycobacterium bovis* can cause tuberculosis in humans clinically indistinguishable from disease caused by *M. tuberculosis*. Public health concerns posed by potential transmission of *M. bovis* from cattle to humans prompted many countries to implement national programmes to eradicate tuberculosis from cattle. Most eradication campaigns have been successful in decreasing the prevalence of bovine tuberculosis. However, in spite of long-standing and costly efforts, some countries have found it impossible to eradicate bovine tuberculosis. One obstacle responsible for many faltering eradication campaigns has been the presence of a wildlife reservoir of

M. bovis infection. In most cases, wildlife originally acquired tuberculosis from cattle; however, the disease is now spilling back from wildlife to cattle, impeding the progress of eradication (Daszak et al., 2000; Miller and Kaneene, 2006). In an effort to mitigate wildlife to cattle transmission of *M. bovis*, some countries are investigating the possible role of vaccination.

In 1994, a free-ranging white-tailed deer (*Odocoileus virginianus*) in Michigan was diagnosed with tuberculosis caused by *M. bovis* (Schmitt et al., 1997). Subsequent surveys identified a focus of *M. bovis* infection in free-ranging white-tailed deer in northeast Michigan (O'brien et al., 2001, 2002). This represented the first known reservoir of *M. bovis* in free-ranging wildlife in the United States and a significant impediment to the ongoing effort to eradicate bovine tuberculosis from domestic livestock. At least 44 cattle herds on 39 different farms (five herds have been infected twice) in Michigan have been diagnosed with tuberculosis since the discovery of tuberculosis in free-ranging deer, presumably from direct or indirect contact with infected deer. Control and surveillance measures have now been in place in Michigan for over 10 years and a significant reduction in apparent prevalence of tuberculosis in deer has been achieved (O'brien et al., 2006). Current disease control measures include decreasing deer density through increased hunting and strict control of feeding and baiting of white-tailed deer. However, hunter support for further population reduction is waning and public resentment of control measures has grown (O'brien et al., 2006). A control measure that could be applied to specific areas of sustained high disease prevalence is vaccination of deer to prevent infection, disease, or transmission. Recently, protection has been demonstrated by parenteral vaccination of white-tailed deer with *M. bovis* bacillus Calmette Guerin (BCG) Pasteur and oral vaccination of white-tailed deer with BCG Danish (Palmer et al., 2007; Nol et al., 2008). *Mycobacterium bovis* BCG was first used as an anti-tuberculosis vaccine in humans in 1921 and is one of the oldest and most widely used vaccines in the world today (Fine, 1989). Since its discovery, propagation of *M. bovis* at various laboratories has resulted in numerous strains that differ morphologically and genetically. Reports of vaccine efficacy in humans vary widely by geographical region and among different age groups; however, consistent protection has only been observed against miliary tuberculosis and tuberculous meningitis in neonates. The great variability in efficacy is attributed to one or more factors including differences in vaccine strains (Fine, 1989).

The purpose of this study was to evaluate the protective efficacy of both BCG Danish and BCG Pasteur administered parenterally using a model of intratonsilar inoculation of white-tailed deer. BCG Danish and Pasteur are both considered late strains in relation to the original attenuated strain produced by Albert Calmette and Camille Guerin; however, these strains have been shown to differ genetically (Brosch et al., 2007). In particular, BCG Danish belongs to a group of BCG strains considered to be the most attenuated (Brosch et al., 2007).

Materials and Methods

Animals, vaccination and challenge

Thirty-five white-tailed deer (~1 year old, 20 castrated males and 15 females) were obtained from a captive breeding herd (tuberculosis- and paratuberculosis-free) at the National Animal Disease Center (Ames, IA, USA). All deer were housed and cared for according to institutional guidelines. Deer were randomly assigned to one of three groups; one dose of 10^7 colony-forming units (CFU) *M. bovis* BCG Pasteur ($n = 12$); one dose of 10^7 CFU *M. bovis* BCG Danish ($n = 11$); or unvaccinated deer ($n = 12$). Deer were vaccinated SC on the right side of the neck, midway between the head and shoulder. One hundred twenty days after vaccination all but 3 deer from each group were inoculated intratonsilarly, as described previously, with approximately 495 CFU of virulent *M. bovis* strain 1315 (NADC designation) into each tonsilar crypt for a total dose of 990 CFU (Palmer et al., 1999). To evaluate vaccine persistence within tissues, 3 non-vaccinated deer and 3 each of BCG Pasteur and BCG Danish vaccinated deer were not challenged, but maintained and examined at the termination of the study (vaccinated, non-challenged deer, $n = 9$).

Strain 1315, used for challenge, was originally isolated from a white-tailed deer in Michigan. For challenge inoculation, deer were anaesthetized by IM injection of a combination of xylazine (2 mg/kg) (Mobay Corporation, Shawnee, KS) and ketamine (6 mg/kg) (Fort Dodge Laboratories, Fort Dodge, IA). After inoculation, the effects of xylazine were reversed by IV injection of tolazoline (4 mg/kg) (Lloyd Laboratories, Shenandoah, IA). Vaccinated and unvaccinated deer were housed together in an outdoor paddock prior to challenge with virulent *M. bovis,* at which time they were moved to appropriate animal housing. Experimental infection was done inside a biosafety level 3 (BL-3) building with personnel wearing appropriate personal protective equipment, including full-face respirators with HEPA filtered canisters to prevent exposure to aerosolized *M. bovis*. The BL-3 animal housing had negative air pressure compared to the outside. Airflow was such that air was pulled out of animal rooms towards a central corridor, preventing air exchange between rooms. Airflow was adjusted to produce 11.4 air changes per hour. Deer were housed 4 per pen and fed a commercial pelleted feed with free access to water.

All procedures were approved by the National Animal Disease Center Institutional Animal Care and Use Committee.

Challenge inoculum and vaccines

The *M. bovis* BCG strains as well as the challenge strain *M. bovis* 1315 were grown in Middlebrook's 7H9 media supplemented with 10% oleic acid-albumin-dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO) as described (Bolin et al., 1997). Mid log-phase growth bacilli were pelleted by centrifugation at 750 × ***g***, washed twice with phosphate buffered saline (PBS) (0.01 M, pH 7.2) and diluted to the appropriate cell density in 2 ml of PBS. Bacilli were enumerated by serial dilution plate counting on Middlebrook's 7H11 selective media (Becton Dickinson, Cockeysville, MD). A single vaccine dose consisted of 10^7 CFU *M. bovis* BCG in 1.5 ml PBS.

Necropsy and tissue sampling

One hundred thirty days after challenge with virulent *M. bovis* (i.e. 250 days after vaccination) all deer were euthanized by IV sodium pentobarbital. At necropsy, the following tissues were collected and processed for isolation of *M. bovis* and microscopic analysis as described (Palmer et al., 2002a): palatine tonsil; lung; liver; mandibular, parotid, medial retropharyngeal, tracheobronchial, mediastinal, hepatic, mesenteric, superficial cervical and prefemoral lymph nodes. Two hundred fifty days after vaccination, vaccinated but not challenged deer were also euthanized and tissues collected as described above for *M. bovis* BCG isolation and microscopic analysis.

Lymph nodes were cross-sectioned at 0.5 cm intervals and examined. Each lung lobe was examined separately and cross-sectioned at 0.5 to 1.0 cm intervals. Lungs and lymph nodes were subjected to semi-quantitative scoring of gross lesions adapted from Vordermeier et al. (Vordermeier et al., 2002). Lung lobes (left cranial, left caudal, right cranial, right caudal, middle and accessory) were subjected to the following scoring system: (0) no visible lesions; (1) no external gross lesions, but lesions seen upon slicing; (2) <5 gross lesions of <10 mm in diameter; (3) >5 gross lesions of <10 mm in diameter; (4) >1 distinct gross lesion of >10 mm in diameter; (5) coalescing gross lesions. Scoring of lymph node gross lesions was based on the following scoring system: (0) no visible lesions; (1) small focal lesion (1–2 mm in diameter); (2) several small foci; (3) extensive lesions. Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin and included all tissues collected for bacteriological examination. For microscopic examination, formalin-fixed tissues were processed by routine paraffin-embedment techniques, cut to 5 μm sections and stained with hematoxylin and eosin (HE). Adjacent sections were cut from samples containing lesions suggestive of tuberculosis (caseonecrotic granulomas) and stained by the Ziehl-Neelsen technique for identification of acid-fast bacteria (AFB). Microscopic lesions were graded (stages I-IV) according to criteria adapted from that described by Rhoades et al. (Rhoades et al., 1997). Stage I (initial) granulomas are characterized by accumulations of epithelioid macrophages admixed with low numbers of lymphocytes and neutrophils. Multinucleated giant cells may be present but necrosis is absent. Acid-fast bacilli, when present, are seen within macrophages or multinucleated giant cells. Stage II (solid) granulomas are characterized by accumulations of epithelioid macrophages surrounded by a thin connective tissue capsule. Infiltrates of neutrophils and lymphocytes may be present at the granuloma periphery as well as multinucleated giant cells. Necrosis when present is minimal. Stage III (necrotic) granulomas are characterized by complete fibrous encapsulation. Necrotic cores are surrounded by a zone of epithelioid macrophages admixed with multinucleated giant cells and lymphocytes. Stage IV (necrotic and mineralized) granulomas are characterized by a fibrous capsule surrounding irregular multicentric granulomas with multiple necrotic cores. Necrotic cores contain foci of dystrophic mineralization. Epithelioid macrophages and multinucleated giant cells surround necrotic areas and there are often moderate to marked infiltrates of lymphocytes. Acid-fast bacilli are often present in moderate numbers and primarily located within the caseum of the necrotic core.

Isolation and identification of mycobacterial isolates

Tissues were processed for isolation of *M. bovis* as previously described (Palmer et al., 2002b). Isolates of *M. bovis* were identified by colony morphology, growth, and biochemical characteristics as well as by PCR. PCR was used to confirm *M. bovis* from tissues collected at necropsy and to distinguish virulent *M. bovis* from BCG Danish or BCG Pasteur. BCG was identified by the absence of RD1 using the primers ET1, ET2 and ET3 from Talbot et al., (1997) following the reaction and thermocycler conditions described below. BCG was further characterized using PCR using a technique similar to that of Talbot et al., (1997) which exploits regions of difference between the strains. BCG strain Danish was differentiated from strain Pasteur using the following primers: RDDen1-CAGGCTAGTTGCCAACACATT (forward); RDDen2-ATGTGTGGGCTGTGTGCTAA (forward); RDDen3-CGCAGGTTAACAGCAGTTTG (reverse). Primer setup and

Table 1. PCR primer scheme and the resulting PCR products used to differentiate BCG strains

	Amplicon size (bp)		
Primers	*M. bovis*	BCG Pasteur	BCG Danish
RDDen1, RDDen3	NP	1090	260
RDDen2, RDDen3	260	260	NP

NP, no product.

interpretation are described in Table 1 using the reaction and thermocycling conditions described below.

Putative mycobacterial isolates visible on Middlebrook 7H10 or Middlebrook 7H11 media were transferred to 25 μl of sterile Tris-EDTA (TE) buffer using a sterile-disposable inoculation loop. When ≤10 colonies were present, all isolates were collected and examined separately. When >10 colonies were present, a minimum of 10% of the colonies present were collected and examined separately. The bacterial suspension was heat inactivated at 80°C for 10 min and heated for an additional 10 min. One microlitre of the bacterial preparation was added to the following PCR master mix: 1× PCR Reaction Buffer with $MgCl_2$ (Roche Applied Science, Indianapolis, IN, USA), 50 pM of each primer, 200 μM of each dNTP (PCR Nucleotide Mix, Roche Applied Science), 2 U FastStart Taq (Roche Applied Science), 5 μg BSA (Ambion, Austin, TX, USA) in a total reaction volume of 50 μl. Touchdown PCR was performed with an initial preheating step of 2 min at 94°C followed by denaturation at 94°C for 45 s, annealing started at 65°C for 1 min with a 1°C decrease per cycle followed by extension at 72°C for 2 min, after the annealing temperature reached 50°C, an additional 29 cycles were performed. A final extension at 72°C for 10 min was included. Samples were analysed by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide.

Isolates of acid fast bacteria that were not identified by PCR as virulent *M. bovis* or *M. bovis* BCG were further identified using 16S ribosomal DNA sequencing as described previously (Kierschner and Bottger, 1998). Sequences were then identified through the use of a mycobacterial species sequence database (Harmsen et al., 2003).

Statistical analysis

Mean group values for lesion scores were compared using an unpaired Student's *t*-test with Welch's correction for non-parametric data to compensate for the lack of homoscedascity (GraphPad Prism, GraphPad Software, San Diego, CA, USA). A *P*-value <0.05 was considered significant.

Results

Mycobacterium bovis challenged deer, vaccinated and non-vaccinated

In non-vaccinated deer, the most common site for lesion development after challenge was the medial retropharyngeal lymph node ($n = 4$) while in BCG Pasteur vaccinated deer, the most common sites for lesions were the medial retropharyngeal and tracheobronchial lymph nodes ($n = 2$ each). In BCG Danish vaccinated deer, lesions were not seen in the medial retropharyngeal lymph node and the most common sites for lesions were the tracheobronchial and mesenteric lymph nodes ($n = 2$ each), followed by lung ($n = 1$).

Gross lesion severity scores of the medial retropharyngeal lymph node individually as well as all lymph nodes examined combined were lower ($P < 0.05$ and $P < 0.1$, respectively), in BCG Danish vaccinated deer than in non-vaccinated deer, but not different from BCG Pasteur vaccinated deer (Fig. 1). In both groups of BCG

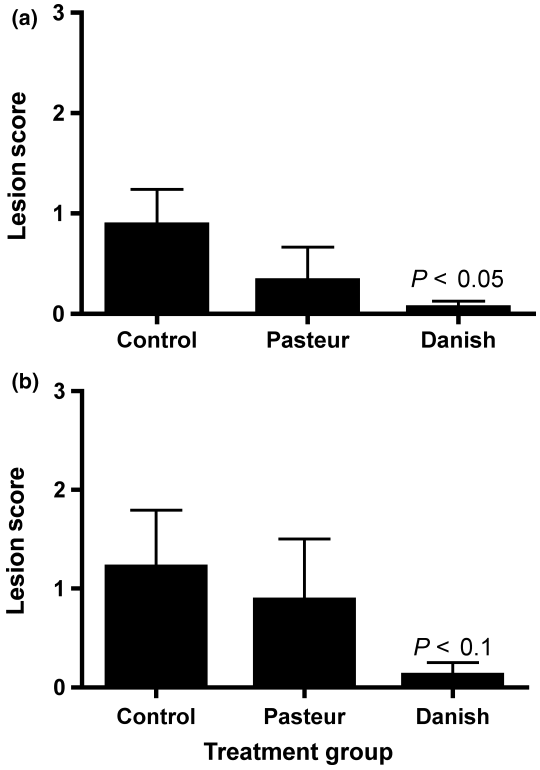

Fig. 1. Gross lesion severity scores from medial retropharyngeal lymph node (a) or all lymph nodes examined combined (b) of unvaccinated deer or deer vaccinated s.c. with 1×10^7 CFU *Mycobacterium bovis* BCG Pasteur or 1×10^7 CFU *M. bovis* BCG Danish and challenged intratonsilarly with 990 CFU of virulent *M. bovis*. Values represent the mean gross lesion severity score ± SE. Scoring system: (0) no visible lesions; (1) small focal lesion (1–2 mm in diameter); (2) several small foci; (3) extensive lesions.

vaccinated deer, lung lesion scores were lower than, but not significantly different from those of non-vaccinated deer (data not shown).

Microscopic evaluation of the medial retropharyngeal lymph nodes revealed a lack of granulomas in BCG Danish vaccinated deer, whereas granulomas of all histological stages (I–IV) were seen in medial retropharyngeal lymph nodes from non-vaccinates and BCG Pasteur vaccinated deer. In the lung, microscopic granulomas of all stages (I–IV) were seen in non-vaccinates. Stage IV microscopic granulomas were not seen in the lungs of BCG Danish or BCG Pasteur vaccinated deer; however, stages I–III were present.

Virulent *M. bovis* was isolated from 6/9 unvaccinated deer, 3/8 Danish vaccinated deer and 2/9 Pasteur vaccinated deer (Table 2). Regardless of treatment group, virulent *M. bovis* was most commonly isolated from the medial retropharyngeal lymph node, followed by the tracheobronchial lymph node, tonsil and lung. BCG Danish was isolated from 4/8 Danish vaccinated deer, with the mediastinal lymph node, tracheobronchial lymph node and superficial cervical lymph nodes being the most common sites of isolation. BCG Pasteur was isolated from the mediastinal lymph node of two BCG Danish vaccinated deer and both the mediastinal and hepatic lymph nodes of a third BCG Danish vaccinated deer (Table 2). Likewise, BCG Pasteur was isolated from 4/9 BCG Pasteur vaccinated deer with the mediastinal lymph node, tracheobronchial lymph node and superficial cervical lymph nodes being the most common sites of isolation. BCG vaccine strains were not isolated from control non-vaccinated deer challenged with *M. bovis*. Non-tuberculous mycobacteria were isolated from 5 deer. *Mycobacterium terrae* was isolated from 1/9 non-vaccinated deer and 1/8 BCG Danish vaccinated deer. *Mycobacterium septicum* was isolated from 1/9 non-vaccinated deer and 1/9 BCG Pasteur vaccinated deer. *Mycobacterium avium* was isolated from 1/9 BCG Pasteur vaccinated deer (Table 2). Lesions were not seen in tissues from which non-tuberculous mycobacteria were isolated. In one BCG Pasteur vaccinated deer, virulent *M. bovis*, BCG Pasteur and *M. avium* were all isolated from a non-lesioned tracheobronchial lymph node.

Table 2. Summary of gross lesions, microscopic lesions and bacteriological isolation of *Mycobacterium bovis* from white-tailed deer vaccinated with either BCG Danish or BCG Pasteur and challenged by intratonsilar inoculation of 990 CFU of virulent *M. bovis*

	Unvaccinated	BCG Danish	BCG Pasteur
Gross lesions	4/9	1/8	2/9
Microscopic lesions	4/9	5/8	3/9
Isolation of virulent *M. bovis*	6/9	3/8	2/9
Isolation of *M. bovis* BCG Danish	0/9	4/8	0/9
Isolation of *M. bovis* BCG Pasteur	0/9	3/8	4/9
Isolation of both BCG Danish and Pasteur from same animal	0/9	1/8	0/9
Isolation of BCG from tissues with lesions	0/9	3/8	1/8
Isolation of non-tuberculous mycobacteria	2/9[a,c]	1/8[a]	2/9[b,c]
Deer from which *M. bovis* was not isolated and lesions were not seen[d]	3/9	3/8	5/9

[a]*M. terrae.*
[b]*M. avium.*
[c]*M. septicum.*
[d]Deer from which neither virulent *M. bovis* nor *M. bovis* BCG was isolated and lesions were not seen.

Vaccinated, non-challenged deer

In vaccinated, non-challenged deer (three from each treatment group, $n = 9$) gross lesions consistent with tuberculosis were not seen (Table 3). In one BCG Danish vaccinated deer microscopic granulomas were seen in the superficial cervical, tracheobronchial and hepatic lymph nodes. In a second BCG Danish vaccinated deer, microscopic lesions were seen in the superficial cervical lymph node only. In one BCG Pasteur vaccinated deer microscopic granulomas were seen in the hepatic lymph node. In all cases, granulomas were of stages II–III (Fig. 2). BCG Danish was recovered from all three Danish vaccinated deer, while BCG Pasteur was recovered from 2/3 Pasteur vaccinated deer. PCR and 16S ribosomal DNA sequencing revealed an isolate from the hepatic lymph

Table 3. Summary of gross lesions, microscopic lesions and bacteriological isolation of *Mycobacterium bovis* BCG from white-tailed deer vaccinated with either BCG Danish or BCG Pasteur and examined 250 days later

	Unvaccinated	BCG Danish	BCG Pasteur
Gross lesions	0/3	0/3	0/3
Microscopic lesions	0/3	2/3[a]	1/3[b]
Isolation of *M. bovis* BCG Danish	0/3	3/3[c]	0/3
Isolation of *M. bovis* BCG Pasteur	0/3	0/3	2/3[d]
Isolation of BCG from tissues with lesions	0/3	1/3	0/3

[a]Superficial cervical lymph node from one deer; tracheobronchial, hepatic and superficial cervical lymph node from a second deer.
[b]Hepatic lymph node from one deer.
[c]Tracheobronchial and mediastinal lymph nodes from one deer; hepatic, superficial cervical and mediastinal lymph nodes from a second deer; superficial cervical lymph node from a third deer.
[d]Superficial cervical lymph node from one deer; lung from a second deer.

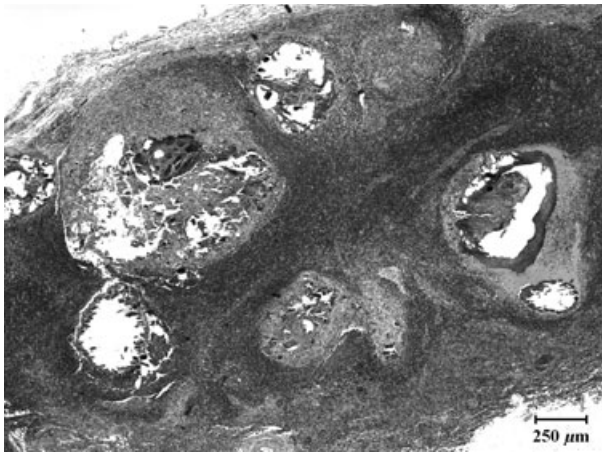

Fig. 2. Section of superficial cervical lymph node from deer vaccinated SC with 1×10^7 CFU of *Mycobacterium bovis* BCG Danish and examined 250 days later. Note vaccine-induced granulomas composed of central necrotic, mineralized cores, epithelioid macrophages and multinucleated giant cells. H/E. Bar = 250 μm.

node of one BCG Danish vaccinated deer to be consistent with an unknown mycobacterial species. Neither BCG vaccine strains nor non-tuberculous mycobacteria were recovered from non-vaccinated, non-challenged control animals.

Discussion

Previous studies have shown that BCG Danish and BCG Pasteur can effectively decrease disease severity in experimentally challenged white-tailed deer (Palmer et al., 2007; Nol et al., 2008). However, a novel finding in this study was persistence of both Danish and Pasteur vaccine strains within host tissues for 250 days after vaccination. Moreover, vaccine-induced microscopic lesions and dissemination of vaccine to multiple sites was seen in at least 1 deer from each vaccinated group. Vaccine persistence within host tissues is an important safety concern in animals that may be consumed for food. In red deer, *M. bovis* BCG Pasteur was still present in tissues from the site of injection as well as the draining lymph nodes in 2/6 and 3/6 deer, respectively, 3 months after s.c. vaccination with 2×10^6 CFU of *M. bovis* BCG Pasteur (Slobbe et al., 1999). Lesions in regional lymph nodes and shedding of vaccine have not been features of studies involving red deer (Griffin et al., 1993) or cattle. In this study, unlike red deer, BCG not only persisted in vaccinated deer, but spread to multiple sites, was associated with microscopic granulomas and in the case of BCG Pasteur vaccinated deer, was shed, transmitted and later isolated from 3/8 deer vaccinated with BCG Danish. It is interesting to note the apparent shedding and transmission of BCG Pasteur, but not BCG Danish. It is possible that this observation reflects increased shedding of vaccine from Pasteur vaccinated deer. Alternatively, both vaccines may be shed, but environmental survivability may be decreased in BCG Danish compared to BCG Pasteur.

Vaccine persistence presents an interesting challenge. While a significant safety concern with food producing animals, persistence of BCG within host tissue is necessary for development of an effective immune response. Studies in mice demonstrate persistence of *M. bovis* BCG for up to 30 weeks and spread to distant organs after s.c. vaccination (Olsen et al., 2004; Aldwell et al., 2006). Murine studies further show that persistence of *M. bovis* BCG is vital in sustaining long-lasting immunological memory. Vaccinated mice receiving chemotherapy to eliminate residual post-vaccinal BCG demonstrated inferior cell-mediated immune responses and inferior protection against challenge with virulent *M. tuberculosis* as measured by colonization of the spleen by *M. tuberculosis* when compared to mice still harboring low numbers of BCG (Olsen et al., 2004; Cross et al., 2007).

Persistence of vaccine strains within tissues also highlights the need for careful identification of mycobacterial isolates in vaccine efficacy studies. Studies relying on bacteriological culture to measure vaccine-induced protection could be misleading if isolates of BCG are mistakenly identified as virulent *M. bovis*. Traditional culture methods, without further biochemical or nucleic acid based analysis cannot differentiate BCG from virulent *M. bovis*.

The importance of careful identification of mycobacterial isolates is further supported by isolation of non-tuberculous mycobacteria from several deer. This finding also illustrates the ubiquitous nature of environmental mycobacteria and their ability to colonize susceptible hosts. Absence of lesions supports their non-pathogenic nature in deer. Isolation of non-tuberculous mycobacteria underscores the challenges posed by exposure to saprophytic non-tuberculous mycobacteria present in the environment. Environmental exposure of red deer or white-tailed deer to environmental saprophytic mycobacteria did not influence susceptibility to experimental infection with *M. bovis* (Griffin et al., 1999; Palmer et al., 2007). The number of affected deer in this study is too low to reach any conclusions concerning a positive or negative effect of colonization with environmental mycobacteria on vaccine efficacy. However, studies in cattle have shown that sensitization of calves to environmental saprophytic mycobacteria adversely affects the protective efficacy of BCG vaccination (Buddle et al., 2002). Similarly, one explanation for the highly variable efficacy observed in human vaccine trials has been exposure to saprophytic environmental mycobacteria (Brandt et al., 2002). Vaccination of neonatal calves has been used as a

strategy to avoid prior sensitization by saprophytic environmental mycobacteria. Indeed, vaccination of neonatal calves induces a higher level of immunity than that seen in calves vaccinated at 5–6 months of age (Buddle et al., 2003a,b; Hope et al., 2005). A similar strategy could be feasible for vaccination of captive white-tailed deer, but would be problematic for vaccination of free-ranging white-tailed deer.

Comparative genomic analysis of numerous BCG strains has demonstrated various deletions and duplications that distinguish one BCG strain from another. Although both BCG Danish and Pasteur are categorized as late strains in relation to the original BCG isolates, BCG Pasteur has deletions in regions RD14 and RD15; regions that are present in BCG Danish. BCG Pasteur is also unique among BCG strains in containing a duplication of a region known as DU1 (Brosch et al., 2007). In this study, gross lesion scores were decreased in both Danish and Pasteur vaccinated deer indicating vaccine induce protection. These findings are consistent with previous work with BCG vaccinated white-tailed deer (Palmer et al., 2007; Nol et al., 2008). Studies in cattle examining the comparative efficacy of BCG Danish and Pasteur showed that both strains protected cattle equally well from intratracheal challenge with virulent *M. bovis* (Wedlock et al., 2007). In this study, some measures of protection suggested BCG Danish provided superior protection to that conferred by BCG Pasteur. Specifically, in the medial retropharyngeal lymph node, BCG Danish prevented the development of advanced granulomas in a fashion superior to that seen in Pasteur vaccinates. Advanced stage IV lesions are characterized by multiple coalescent granulomas with extensive necrosis and numerous intralesional acid-fast bacilli (Palmer et al., 2007). Such advanced lesions increase the likelihood of disease dissemination within the host and disease transmission between hosts. A vaccine that prevents advanced lesion development, while not preventing infection or disease, is likely to decrease bacterial shedding and disease transmission. In the intratonsilar model of inoculation used in this study, the most common site for lesion development is the medial retropharyngeal lymph node (Palmer et al., 1999, 2002a, 2007). Therefore, it is particularly interesting that Danish vaccinates did not develop lesions in the medial retropharyngeal lymph node. A similar finding was observed in a recent study of BCG Danish vaccination in white-tailed deer (Nol et al., 2008). Protection against experimental challenge has also been observed in BCG Danish vaccinated cattle (Wedlock et al., 2007). Given the demonstrated efficacy and the fact that BCG Danish is currently approved for use in humans (Wedlock et al., 2007), BCG Danish may afford a viable option for vaccination of deer.

It is doubtful that parenteral vaccination, such as that used in this study, would be possible with free-ranging wildlife on a large scale. More likely, an oral vaccine such as that currently used to vaccinate wildlife against rabies would be necessary (Desmettre et al., 1990). Oral delivery of BCG, while posing several challenges, has been shown to be feasible and efficacious with white-tailed deer (Miller et al., 1999; Nol et al., 2008). Lipid based preparations to protect live *M. bovis* BCG from the harmful effects of the gastric environment have proven effective in brushtail possum and white-tailed deer vaccination trials (Aldwell et al., 2003; Nol et al., 2008).

Although the ultimate goal of any tuberculosis vaccination programme is to eradicate tuberculosis, a more immediate aim is to reduce the rate of transmission between susceptible hosts. Any tuberculosis vaccine considered for use in free-ranging white-tailed deer need not protect animals against infection or disease. Disease transmission and prevalence would decline if lesion severity and bacterial shedding were decreased in vaccinated deer. This study demonstrated reduced lesion severity in white-tailed deer vaccinated with either BCG Danish or BCG Pasteur and challenged with virulent *M. bovis*; however, superior protection against advanced lesion formation was seen in BCG Danish vaccinates.

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