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ORIGINAL ARTICLE

Persistence of *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) Danish In White-tailed Deer (*Odocoileus virginianus*) Vaccinated with a Lipid-Formulated Oral Vaccine

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Summary

Mycobacterium bovis, the causative agent of tuberculosis in animals, has a broad host range, including humans. Historically, public health concerns prompted programs to eradicate tuberculosis from cattle in many nations. Eradication efforts decreased the prevalence of bovine tuberculosis; nevertheless, some countries encountered significant obstacles, not least of which was a wildlife reservoir of *M. bovis*. Efforts to decrease the size of the affected wildlife populations have neither eliminated disease nor eliminated transmission to cattle. Consequently, the use of a vaccine for wildlife is being explored. The vaccine most studied is *M. bovis* BCG, an attenuated live vaccine, first developed 100 years ago. The most efficient and effective means of vaccinating wildlife will be an oral vaccine. White-tailed deer in Michigan, USA, constitute a reservoir of *M. bovis*. White-tailed deer are a popular game species, and as such, represent a food animal to many hunters. BCG persistence in deer tissues could result in human exposure to BCG. Although non-pathogenic, BCG exposure could induce false-positive skin test results, confounding the central component of public health surveillance for TB. The objective of the present study in white-tailed deer was to evaluate persistence of lipid-encapsulated BCG and a liquid suspension of BCG after oral administration at two different dosages. Vaccine was not recovered at any time after oral consumption of a bait containing a single dose (1×10^8 CFU) of lipid-encapsulated BCG. However, persistence was consistent in deer consuming 10 lipid-encapsulated baits (1×10^9 CFU), with BCG recovered from at least one deer at 1, 3, 6, 9 and 12 months after consumption. Persistence of up to 9 months was seen in deer vaccinated with orally with a liquid suspension. Persistence of BCG was limited to lymphoid tissue and never found in samples of muscle collected at each time point. Although the risk of exposure to hunters is low, BCG persistence should be considered prior to field use in white-tailed deer.

Introduction

Mycobacterium bovis is the causative agent of tuberculosis in cattle and an important zoonotic pathogen. It has a broad host range, including most mammals, and can cause tuberculosis in humans that is clinically indistinguishable from disease caused by *Mycobacterium tuberculosis*. Historically, public health concerns posed by potential transmission of *M. bovis* from cattle to humans prompted many countries to implement national programs to eradicate tuberculosis from cattle. Eradication campaigns have generally been successful in decreasing the prevalence of bovine

tuberculosis; nevertheless, some countries have encountered significant obstacles. One factor responsible for faltering eradication campaigns has been the presence of a wildlife reservoir of *M. bovis*. In most cases, wildlife originally acquired tuberculosis from cattle; however, *M. bovis* 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 reduce wildlife to cattle transmission of *M. bovis,* some countries are investigating the possible role of wildlife vaccination (Aldwell et al., 1995; Ballesteros et al., 2009). Oral administration of a vaccine is the most practical and cost-effective means of vaccinating wildlife. Illustratively, oral rabies vaccines have been used to successfully vaccinate foxes, raccoons and other wildlife against rabies (MacInnes et al., 2001; Rosatte et al., 2007).

In 1994, a hunter-killed white-tailed deer (*Odocoileus virginianus*) in Michigan was diagnosed with tuberculosis due to *M. bovis* (Schmitt et al., 1997). Subsequent surveys identified a focus of *M. bovis* in free-ranging white-tailed deer in north-east Michigan (O'Brien et al., 2001, 2002). This represented the first known reservoir of *M. bovis* in free-ranging wildlife in the United States (US), the first known epizootic of tuberculosis in white-tailed deer and a significant impediment to the US effort to eradicate bovine tuberculosis from livestock. Disease control measures in Michigan included, among other policy changes, decreasing deer density through increased hunting. 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). However, public support for further population reduction is waning (O'Brien et al., 2006). Vaccination of deer in specific areas of sustained higher disease prevalence could be used to prevent infection, disease or transmission. Recently, protection, in the form of decreased disease severity, was demonstrated by subcutaneous (SC) and oral vaccination of white-tailed deer with *M. bovis* bacillus Calmette–Guérin (BCG) (Palmer et al., 2007, 2009; Nol et al., 2008). These studies also demonstrated BCG persistence within host tissue for up to 9 months after vaccination as well as shedding of viable BCG to non-vaccinated deer.

White-tailed deer hunters frequently consume venison (Wilkins et al., 2003); consequently, the persistence of BCG in vaccinated deer presents a potential public health concern. Although BCG is generally considered avirulent for immunocompetent humans, exposure to BCG could cause false-positive responses to the tuberculin skin test, interfering with public tuberculosis surveillance efforts. Previous studies have shown BCG persistence in white-tailed deer for up to 3 and 9 months after oral or SC vaccination, respectively (Palmer et al., 2010).

Lipid-formulated baits have been developed for oral delivery of BCG. Lipid encapsulation protects viable BCG from gastric degradation, allowing lipolytic enzymes of the small and large intestines to liberate BCG, enhancing uptake by gastrointestinal-associated lymphoid tissue (GALT) (Aldwell et al., 2003b). Lipid-encapsulated BCG delivered orally to brushtail possums (*Trichosurus vulpecula*) persist in mesenteric lymphoid tissue of the GALT for up to 8 weeks (Wedlock et al., 2005). Possums vaccinated with lipid-encapsulated BCG shed viable vaccine in faeces for up to 7 days after vaccination, but always in low numbers [$<10^3$ colony-forming units (CFU)/gm faeces] (Wedlock et al., 2005). Both vaccine persistence and shedding are important as non-target species such as scavengers, predators and even cattle may be exposed to BCG in the environment as well as persistent BCG in tissues. Experimental vaccination and challenge studies in brushtail possums, a wildlife reservoir host of *M. bovis* in New Zealand, showed decreased weight loss, lower lung–body weight ratio, fewer extrapulmonary lesions, and lower lung and spleen bacterial counts in vaccinates compared to non-vaccinates (Cross et al., 2009). Field vaccination studies, using lipid-encapsulated BCG, show protection against natural infection with an estimated efficacy of 95–96% (Ramsey et al., 2009; Tompkins et al., 2009). The objective of the present study in white-tailed deer was to evaluate persistence of lipid-encapsulated BCG after oral administration at two different dosages, as well as a liquid suspension of BCG.

Materials and Methods

Vaccine

Mycobacterium bovis BCG Danish was used as the vaccine strain. Bacteria were grown to mid-log phase in 175-ml flasks (Falcon) containing Middlebrook 7H9 medium (Difco, Detroit, MI, USA) supplemented with albumin–dextrose–catalase (Buddle et al., 1995). Bacilli were harvested by centrifugation and washed twice in phosphate-buffered saline (PBS), 0.01 M, pH 7.2, prior to storage at −70°C. The CFU of BCG was determined retrospectively by plating on 7H11 agar (Difco) supplemented with oleic acid–albumin–dextrose–catalase (Becton Dickinson, Cockeysville, MD, USA) as described previously (Buddle et al., 1994).

Lipid composition

A fractionated lipid product was previously selected for encapsulation of BCG on the basis of melting temperature (32°C) and the ability to maintain bacilli in a viable and uniform suspension as assessed by counting CFU (Aldwell et al., 2003b). The lipid product consisted of triglycerides of fatty acids comprising 1% myristic acid, 25% palmitic acid, 15% stearic acid, 50% oleic acid and 6% linoleic acid (Aldwell et al., 2003a,b).

Lipid encapsulation of BCG

Glucose (10 mg/ml), monosodium glutamate (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) and 10% vol/vol albumin–dextrose–catalase were added to the lipid formulation. These additives were dispersed with the lipid in the liquid phase (40°C) and were previously shown not to affect BCG viability. Pelleted BCG was resuspended in the liquefied formulation medium that had been warmed to 40°C. The BCG–lipid formulation was transferred to plastic moulds and allowed to solidify with gentle mixing at 4°C. BCG–lipid formulations were removed from the moulds as required for viability testing and vaccination studies (Aldwell et al., 2003a). A single lipid-encapsulated BCG bait contained 1×10^8 CFU BCG.

For the liquid suspension vaccine, mid-log-phase growth bacilli were pelleted by centrifugation at $750 \times g$, washed twice with PBS and diluted to the appropriate cell density in 1.5 ml of PBS. Bacilli were enumerated by serial dilution plate counting on 7H11 agar (Becton Dickinson) (Aldwell et al., 2003a,b). A single vaccine dose consisted of 1×10^8 CFU *M. bovis* BCG in 1.5-ml PBS.

Vaccination of deer

Forty-five white-tailed deer (1-year old, 23 females and 22 castrated males) were obtained either from a tuberculosis-free captive breeding herd at the National Animal Disease Center (Ames, Iowa, USA) or a private tuberculosis-free captive deer facility. All deer were housed and cared for according to institutional guidelines. Additionally, the institutional animal care and use committee as well as the institutional biosafety committee approved all procedures prior to implementation. Deer were randomly assigned to one of three groups of 15 deer each; single oral liquid suspension of BCG, 1×10^8 CFU, (liquid group), single oral lipid-encapsulated BCG bait, 1×10^8 CFU, (1× group) and 10 oral lipid-encapsulated BCG baits; total dose 1×10^9 CFU (10× group). Delivery of the liquid suspension of BCG was carried out as previously described (Nol et al., 2008). Lipid-encapsulated baits were offered to deer while individually housed to ensure consumption. To enhance palatability, baits were mixed with a small amount of shelled corn and molasses.

Necropsy and sample collection

Three deer from each group were euthanized by IV sodium pentobarbital and examined 1, 3, 6, 9 and 12 months after vaccination. At necropsy, the following tissues were collected: palatine tonsil, mandibular, parotid, medial retropharyngeal, tracheobronchial, mediastinal, hepatic, duodenal, proximal jejunal, middle jejunal, distal jejunal, ileal, colic and iliac lymph nodes, and lung. All samples were

processed for isolation of BCG and microscopic analysis as described (Palmer et al., 2002a,b; Hines et al., 2006). Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin, processed by routine paraffin-embedment techniques, cut in 5 μm sections and stained with haematoxylin and eosin (HE). Adjacent sections were cut from samples containing lesions suggestive of tuberculosis (granulomas) and stained by the Ziehl–Neelsen technique for identification of acid-fast bacteria (AFB). For bacteriologic isolation of BCG, samples were submitted pooled into four different groups: head pool (palatine tonsil, mandibular, parotid and medial retropharyngeal lymph nodes), thoracic pool (tracheobronchial and mediastinal lymph nodes and lung), abdominal pool (hepatic, duodenal, proximal jejunal, mid-jejunal, distal jejunal, ileal, colic and iliac lymph nodes) and muscle pool (epaxial, sublumbar, supraspinatus, triceps, semimembranosus, semitendinosus and biceps femoris).

Isolation and identification of mycobacterial isolates

Tissues were processed for isolation of BCG as previously described (Hines et al., 2006) using both the BACTEC 460 radiometric system and BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system (Becton Dickinson). Isolates of BCG were identified by a combination of Ziehl–Neelsen acid-fast staining, nucleic acid probes (AccuProbe, Gen-Probe, San Diego, CA, USA), spoligotyping and a BCG differential PCR described previously (Huard et al., 2003). Further identification of atypical mycobacteria was carried out using partial 16S ribosomal sequencing of the ribosomal polymerase b-subunit as described previously (Kierschner and Bottger, 1998; Ben Salah et al., 2008). Sequences were then identified through use of a mycobacterial species sequence database and GenBank (Harmsen et al., 2003).

Results

Mycobacterium bovis BCG was not isolated from any tissue at any time point from deer consuming a single dose of lipid-encapsulated BCG. However, BCG from the liquid suspension vaccine was recovered from one of three deer each at 1, 6 and 9 months after vaccination (Table 1). Persistence of BCG was most consistent in deer consuming 10 lipid-encapsulated BCG baits (10× group). *Mycobacterium bovis* BCG was recovered from at least one of three deer at each time point, with BCG recovered from two of three and three of three deer at 1 month and 6 months, respectively, after vaccination.

Regardless of group, BCG was most commonly isolated from the head and thoracic tissue pools with fewer isolations from the abdominal tissue pool. Gross lesions

Table 1. Isolation of *Mycobacterium bovis* BCG from deer orally vaccinated with a single dose (1×, 1×10^8 CFU) or 10 doses (10×, 1×10^9 CFU) of a lipid-encapsulated BCG bait or a liquid suspension of *M. bovis* BCG (Liquid, 1×10^8 CFU). Three deer each were euthanized and examined at 1, 3, 6, 9 and 12 months after vaccination

	Months after Vaccination Isolation of *Mycobacterium bovis* BCG				
Group	1	3	6	9	12
1× Oral	0/3	0/3	0/3	0/3	0/3
10× Oral	2/3[a]	3/3[b]	1/3[c]	1/3[d]	1/3[e]
Liquid	1/3f	0/3	1/3[g]	1/3[h]	0/3

[a]Isolation of *M. bovis* BCG from head pool of one deer and abdominal pool of a second deer.
[b]Isolation of *M. bovis* BCG from thoracic pool from two deer and head pool from a third deer.
[c]Isolation of *M. bovis* BCG from head and abdominal pools and hepatic lymph node.
[d]Isolation of *M. bovis* BCG from thoracic and abdominal pools.
[e]Isolation of *M. bovis* BCG from head and abdominal pools.
[f]Isolation of *M. bovis* BCG from head pool.
[g]Isolation of *M. bovis* BCG from thoracic pool.

Table 2. Isolation of non-tuberculous (NTM) or atypical mycobacteria from deer vaccinated orally with lipid-encapsulated *Mycobacterium bovis* BCG (1× and 10× groups) or liquid suspension of BCG (Liquid group)

Isolate	No. of deer[a]	Isolation of *Mycobacterium bovis* BCG[b]
Mycobacterium kansassi	9	3
Mycobacterium avium complex	7	1
Mycobacterium avium subsp. *avium*	1	0
Mycobacterium avium subsp. *hominissuis*	3	0
Mycobacterium intracellulare	4	0
Mycobacterium fortuitum	2	0
Unidentified mycobacterial species	3	0
Mycobacterium smegmatis	1	1
Mycobacterium chelonae	1	0
Mycobacterium setense	1	1
Undetermined *Mycobacterium tuberculosis* complex (likely BCG)	1	0

[a]Number of deer from which the isolate was obtained.
[b]Number of deer from which both BCG and NTM or atypical isolates were isolated.

suggestive of tuberculosis were not seen in any deer from any group at any time point. Microscopic examination revealed histocompatible lesions (granulomas with AFB) in the middle jejunal lymph node of one deer in the 10× dose group at 3 months after vaccination and the medial retropharyngeal lymph node of a separate deer in the 10× dose group at 9 months after vaccination. Lesions were characterized by granulomas composed of epithelioid macrophages, lymphocytes and multinucleated giant cells. Neither central necrosis nor circumferential fibrosis was present. Acid-fast bacteria were rare; nevertheless, when present, they were found within multinucleated giant cells or macrophages. In addition, from the deer with microscopic lesions, BCG was isolated from the corresponding pools of tissues, abdominal and head, respectively.

Ten different non-tuberculous mycobacteria (NTM), atypical mycobacterial species or undetermined *M. tb* complex species were isolated from deer in the current study (Table 2). Bacteria of the *Mycobacterium avium* complex (comprised of numerous subspecies of *M. avium* and *Mycobacteria intracellulare*) were most frequently isolated followed by *Mycobacterium kansasii*. Neither gross nor microscopic lesions were present in tissues from which NTM, atypical mycobacteria or undetermined mycobacterial species were isolated. In 6 of 33 deer, both NTM and BCG were isolated (Table 2). Non-tuberculous mycobacteria were isolated from roughly equal numbers of deer in each of the three treatment groups and at each time point (data not shown).

Discussion

Persistence of BCG in vaccinated animals (deer and cattle) is an important public safety concern as many such animals are used for food. Few studies have examined the persistence of BCG in food animals. Previously in red deer (*Cervus elaphus*) vaccinated SC, BCG Pasteur was recovered from 50% of vaccinates at the site of injection and associated lymph nodes 3 months after vaccination (Slobbe et al., 1999). Importantly, quantitative bacteriological culture demonstrated that BCG was present in very small numbers (i.e. approximately 0.1% of original vaccine dose) (Slobbe et al., 1999). In white-tailed deer, BCG was recoverable up to 9 months after SC injection of 1×10^7 CFU BCG Pasteur (Palmer et al., 2007). Similarly, persistence of up to 9 months was documented in white-tailed deer vaccinated SC with 1×10^7 CFU of either Pasteur or Danish strains of BCG. In a separate study, oral vaccination of white-tailed deer with a liquid suspension of 1×10^9 CFU BCG Danish resulted in persistence of up to 3 months (Palmer et al., 2010). It is clear that BCG persistence should be considered prior to field use in white-tailed deer.

Previously, oral dosing of BCG in liquid suspension resulted in persistence of up to 3 months in white-tailed deer (Palmer et al., 2010). Similar dosing (1×10^8 CFU) in the present study resulted in persistence of up to 9 months. Additionally, in the present study, a greater dose (1×10^9 CFU) of lipid-encapsulated BCG arising from consumption of 10 BCG baits resulted in persistence of up to 12 months. Persistence has also been demonstrated in

murine studies where oral vaccinates using non-encapsulated BCG was 3 months (16/38 animals), while oral dosing with lipid-encapsulated BCG demonstrated extended persistence of up to 7.5 months in all (38/38) animals (Aldwell et al., 2006). Of significant note is that some degree of persistence is necessary to mount an effective immune response to BCG vaccination as shown in murine studies demonstrating that BCG persistence is vital in development of lasting immunological memory (Olsen et al., 2004; Cross et al., 2007). The ideal duration of persistence for maximum host protection and minimum risk to humans consuming BCG vaccinated food animals remains to be determined.

Lipid formulation of BCG protects the live vaccine from the harmful effects of the gastric environment (i.e. acidic pH, enzymes), allowing viable BCG access to important sites of intestinal mucosal immune induction (Aldwell et al., 2006). Previous studies in brushtail possums (*Trichosurus vulpecula*) showed the efficacy of BCG could be improved by protection from gastric degradation through lipid encapsulation or intraduodenal administration (Buddle et al., 1997). Thus far, most use of lipid-encapsulated BCG has been in monogastric omnivorous mammals such as the Eurasian badger (*Meles meles*) and brushtail possum (Aldwell et al., 2003a, 2006; Cross et al., 2009; Corner et al., 2010; Aznar et al., 2011). In contrast, deer are herbivorous ruminants that ferment, absorb and digest plant material in the rumen. The rumen contains a complex resident microbiota of bacteria, protozoa, archaea and fungi. In contrast to the monogastric stomach with a pH < 4, the rumen has a pH of 5.5–6.5 and lacks digestive enzymes of the stomach. The rumen therefore is a vastly different environment than the monogastric stomach. The effect of rumen environment on survivability of BCG is unclear. Furthermore, ruminants regurgitate a food bolus (cud) for rechewing. The process of repeated regurgitation and rechewing is known as rumination. As orally vaccinated ruminants ruminate, oropharyngeal lymphoid tissue (e.g. tonsils) may be repeatedly exposed to BCG. The effect or importance of the rumen environment or multiple BCG exposures on the immune response and host protection is unclear.

In the present study, viable BCG was recovered with equal frequency from the head and thoracic pools. Pooled tissues from the head included mandibular, parotid and medial retropharyngeal lymph nodes as well as palatine tonsil. Mandibular, parotid and medial retropharyngeal lymph nodes all receive afferent lymphatics from tissues of the oral cavity (e.g. tongue, hard and soft palates, oral mucosa and floor of the mouth). Additionally, the medial retropharyngeal lymph node receives afferent lymphatics from the tonsils (Saar and Getty, 1975). As tissues from the head were pooled, it is not possible from the current study to determine which of the pooled head tissues was most commonly affected. However, it is clear that in naturally or experimentally infected deer, the medial retropharyngeal lymph node is the most common site for tuberculous lesion development (Schmitt et al., 1997; Palmer et al., 1999, 2002a; O'Brien et al., 2001). Furthermore, *M. bovis* is found, even in the absence of lesions, in 76% of palatine tonsils from naturally infected animals that have tuberculous lesions in one or more head lymph nodes (Palmer et al., 2002c). Accordingly, it would be reasonable to assume that isolation of BCG from the medial retropharyngeal lymph nodes and tonsils of orally vaccinated deer would also be common.

Presence of BCG in thoracic samples suggests lymphatic or haematogenous spread of BCG after oral vaccination. Although BCG dissemination has been reported previously in white-tailed deer (Palmer et al., 2010), such has not been the case in studies of red deer or cattle (Griffin et al., 1993). Moreover, neither gross nor microscopic lesions have been associated with BCG vaccination studies in red deer or cattle (Griffin et al., 1993).

The isolation of numerous NTM and atypical mycobacteria from white-tailed deer is consistent with previous findings (Palmer et al., 1999, 2001, 2007, 2010). With the exception of *M. avium* subsp. *paratuberculosis* (Mackintosh et al., 2004) and rare cases of disease caused by *M. avium* subsp. *avium* (de Lisle and Havill, 1985) or *M. kansasii* (Hall et al., 2005), most NTM are considered non-pathogenic in deer. The absence of lesions in NTM-infected tissues in the present study would support their non-pathogenic nature in white-tailed deer.

The presence and persistence of BCG in vaccinated deer seemingly implies the potential for human exposure to BCG through consumption of venison. Notwithstanding, persistent BCG has only been found in lymphoid or intestinal organs that are generally avoided as food. Conversely, BCG has not been recovered from muscle (meat) samples commonly consumed as food (Palmer et al., 2010). Moreover, surveys in Michigan, USA, show that the majority of hunters cook venison prior to consumption (Wilkins et al., 2003). Thorough cooking at 60°C for 6 min has been shown to kill virulent *M. bovis* (Merkal and Whipple, 1980). Therefore, potential for human exposure to BCG through venison consumption is low.

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5

the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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