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ORIGINAL PAPER

Testing a molasses-based bait for oral vaccination of white-tailed deer (*Odocoileus virginianus*) against *Mycobacterium bovis*

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Abstract White-tailed deer (*Odocoileus virginianus*) in Michigan, USA, are wildlife reservoirs of bovine tuberculosis (bTB) with documented spread to cattle. In vaccine efficacy trials, *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) administered orally reduces colonization and bTB-associated lesions in white-tailed deer after experimental challenge with virulent *M. bovis*. The objective of this study was to develop and evaluate the palatability of a molasses-based bait for oral delivery of BCG to white-tailed deer. Relevant practical properties of the bait such as physical stability under various environmental conditions were evaluated, as well as palatability. Captive deer consumed baits within 3 h of introduction during 48 of 50 trials. Digital game cameras revealed consumption of all placed baits by one deer over 62 % of the time. Addition of BCG vaccine did not negatively impact palatability. Physical stability analysis demonstrated that ice and water significantly reduced bait stability as measured with a compression assay. Storage of BCG-containing baits at 4 °C showed a slight decrease in colony-forming units (CFUs) by day 31. In contrast, storage at −20 or −80 °C over the same 31-day period showed no significant decrease in BCG viability. The results of this study suggest that molasses-based baits, as prepared here, represent a plausible means of oral delivery of BCG to white-tailed deer under most environmental conditions.

Keywords Bait · White-tailed deer · *Mycobacterium bovis* · Tuberculosis · Vaccination

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Introduction

Mycobacterium bovis is a zoonotic pathogen found around the world that poses a threat to livestock, wildlife, and humans. It is the cause of bovine tuberculosis (bTB) and has been isolated from both dairy and beef cattle herds and wild and captive cervid populations (Nelson, 1999). Human infection may result in disease that is clinically indistinguishable from infection with *Mycobacterium tuberculosis*. Public health concerns have prompted many countries to establish eradication/control programs for *M. bovis* in cattle and captive cervids. These programs have been variably successful, but it has become clear that countries with infected wildlife face greater challenges as wildlife acts as a reservoir of infection for cattle with resultant wildlife-to-cattle transmission. Eradication of tuberculosis in cattle cannot succeed unless *M. bovis* is eliminated from wildlife or wildlife-to-cattle transmission is prevented. In northeastern Michigan, there is a focus of *M. bovis* infection in white-tailed deer. Infected deer have been implicated as the source of infection in over 57 cattle herds from 1988 through spring 2013 (Bill Hench, APHIS TB program staff, personal communication). In the affected area of Michigan, control measures, including decreasing deer density through increased hunting allowances, have resulted in a decrease in disease prevalence in deer from 4.9 to 1.8 % since 1995, but prevalence continues to remain at approximately 2 % (O'Brien et al. 2011; Carstensen et al. 2011). Public acceptance of further decreases in deer densities through increased hunting is waning, and additional or alternative approaches are needed.

Oral vaccination of wildlife has been demonstrated to be an effective form of disease control in multiple species (Ballesteros et al. 2009a; Nol et al. 2008; Hermann et al. 2011, Slate et al. 2009). Delivery of the ONRAB(®) vaccine in baits has been used widely across the USA and Canada to control rabies in raccoons (*Procyon lotor*) (Roess et al. 2012, Slate et al. 2009). Experimentally, oral vaccination of white-tailed deer with the human vaccine, bacillus Calmette–Guerin (BCG), an attenuated strain of *M. bovis*, effectively reduces the severity of disease upon challenge with virulent *M. bovis* (Palmer et al. 2007; Nol et al. 2008). Oral vaccination of deer with BCG represents a possible control strategy in areas of highest prevalence, to reduce deer-to-deer and deer-to-cattle transmission of *M. bovis*. However, effective vaccination requires an effective means of delivery to the target host, such as through oral baits. A number of factors must be considered in identification or development of a bait delivery system. Some of these factors include knowledge of host feeding behavior, seasonal or geographical effects on bait composition, vaccine stability under various environmental conditions, and attractiveness and palatability of bait to the target host (Monaghan 1984).

Previously, free-ranging deer found liquid bait composed of apple juice, water, glycerin, salt, and apple odor, palatable and a plausible means of delivering pharmaceutical agents (Hakin et al. 1996). Dry, shelled corn has been used as an attractant in a topical acaricide delivery device designed for white-tailed deer (Pound et al. 2000). Commercially produced as well as homemade mineral blocks are used to attract deer for wildlife viewing or hunting purposes. Such blocks contain one or several ingredients believed to attract deer, such as molasses, corn, and salt. The purpose of this study was to develop and evaluate the palatability of a molasses-based bait for oral delivery of BCG to white-tailed deer. It was hypothesized that bait delivery would be most effective during the winter months when food sources are depleted, encouraging consumption of nontraditional nutrients. As such, physical stability tests were performed at different temperature and moisture levels relevant for deployment during the winter in Michigan. In addition to bait physical stability, survivability of BCG at varied temperatures and durations of storage was determined using baits that had been inoculated with appropriate dosages of BCG. Bait palatability was determined from observational studies of captive white-tailed deer using both sham and BCG-inoculated baits.

Materials and methods

Bait composition

Molasses baits were prepared by weight using 47.47 % all natural whole wheat flour (Gold Medal, General Mills Sales, Inc., Minneapolis, MN, USA), 27.46 % cane molasses, 11.88 % light brown sugar (Best Choice, Wholesale Grocers, Inc., Kansas City, KS, USA), 8.63 % water, 3.74 % shortening (Crisco, Orrville, OH, USA), 0.49 % sodium bicarbonate, and 0.33 % sodium chloride. Dry ingredients were combined prior to addition of shortening, water, and molasses. Once thoroughly mixed, bait portions were adjusted to 8.0± 0.08 g. Baits measured approximately 1.5 cm high by 3.4 cm in diameter (Fig. 1), small enough for deer to consume in one piece, decreasing the risk of baits falling apart and deer not receiving the desired dose of BCG. Baits were baked at 65 °C for 40 min and allowed to cool to room temperature before storage at 4 °C. Desiccant packets were added to storage bags to reduce moisture content during refrigeration.

Physical stability test

A physical stability test evaluated factors that may affect bait stability when placed in various environments, as described for *Escherichia coli*-laden baits intended for wild boar (Ballesteros et al. 2009a). The baits ($n=72$) were equally distributed to four treatment groups based on temperature including −20, 4, 17, and 37 °C. An additional group ($n=18$, 17 °C) was partially submerged in water while another ($n=18$, −1 °C) was placed in a container of crushed ice. To quantify stability, bait compression was analyzed using three baits from each of the temperature-

Fig. 1 Images of baits measuring 1.5 cm tall by 3.4 cm in diameter

defined and moisture-defined treatment groups. Baits were placed under a 300-g weight for 10 min at 9, 24, 33, 48, 57, and 72-h intervals. Compression was quantified by measuring bait thickness of a mean of three measurements before and after application of the weight.

Mycobacterium bovis viability test

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 oleic–albumin–dextrose–catalase (OADC, DIFCO) (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 colony-forming units (CFUs) of BCG were determined retrospectively by plating on 7H11 agar plates (Becton Dickinson, Cockeysville, MD) as described previously (Buddle et al., 1994).

BCG Danish is known to provide protection against bTB in experimentally infected deer, is commercially available, and is frequently used in vaccine efficacy trials with other host species (Palmer et al. 2009; Carter et al. 2012; Tompkins et al. 2013; Ballesteros et al. 2009b). Aseptically prepared baits were cooled in a biological safety cabinet following baking. Baits were inoculated with 0.025 ml of BCG suspension in four locations for a total of 0.1 ml per bait and then frozen at −80 or −20 °C or refrigerated at 4 °C for at least 72 h. Baits were processed for mycobacterial isolation at days 3, 17, and 31 following inoculation. Three baits per temperature treatment were processed for mycobacterial isolation at each time period.

Each bait was cut in half, and both halves were placed in a 50-ml conical tube containing 15 ml of Middlebrook 7H9 broth with OADC for 10 min, vortexing occasionally for an even distribution of BCG in the diffuse bait suspension. One milliliter of the BCG-bait suspension was added to 9 ml of Middlebrook 7H9 broth with OADC. Quantitative determination of BCG concentrations was evaluated by 10-fold serial dilutions on Middlebrook 7H11 selective agar plates with OADC, and antimicrobial agents including polymyxin B, carbenicillin, amphotericin B, and trimethoprim lactate after 5 weeks at 37 °C. Data on survivability were analyzed with GraphPad Prism 4 (La Jolla, CA), using a Kruskal–Wallis nonparametric ANOVA with Dunn's multiple comparison posttest.

Observations of bait consumption

The rate at which baits were consumed as well as the number of deer eating baits was evaluated in a captive population of white-tailed deer at the National Animal Disease Center (Ames, IA). All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee. Deer received pelleted maintenance feed daily in a feed trough and alfalfa hay ad libitum. Four pens consisted of a three-sided concrete shelter (7 m×10 m) with access to a sand/gravel-based outdoor enclosure (12.5 m×10 m). One of the four pens had access to an approximate 2-acre pasture. Three pens contained three deer of mixed sexes while the fourth pen with access to the pasture contained four bucks. The fifth pen contained approximately 50 deer housed on a pasture. Seven baits per pen were placed on top of the maintenance feed for a total of 35 baits fed per day. Daily consumption was observed for 10 days. Each pen was considered one trial per day, for a total of 50 trials over ten consecutive business days.

Bait consumption was observed using digital game cameras (RECONYX, Inc., Holmen, Wisconsin, USA), mounted 3 m above the feed troughs (Online resource 1). Close observation of unique markings and varied ear tag locations helped distinguish between individual deer. Analysis of the near video images placed consumption into seven categories: "100 % consumption by one deer" referred to all of the baits being consumed by one deer within the same time frame, "multiple deer at feeder" when there were multiple deer in the camera frame at once consuming baits, "same deer returning" in which it was evident through identification that the same deer returned to eat more baits at a later time, "unidentifiable deer returning" indicated a deer returned to consume more baits but was unable to be identified as a previously observed deer, "different deer returning" indicated that deer were able to be identified and distinguished from each other when they returned to eat baits, "multiple deer at feeder and unidentifiable deer returning" referred to multiple deer in the initial camera frame eating baits but was unable to identify the deer that returned, and "multiple deer at feeder and different deer returning" meant there were multiple deer in the initial camera frame eating baits and the deer that returned were able to be distinguished from those that initially ate baits.

Palatability of baits inoculated with BCG

Preliminary bait trials using other bait matrices, such as lipid-formulated pellets or alfalfa, demonstrated that white-tailed deer may be able to detect BCG in baits (unpublished observations) making them less palatable. Therefore, five baits containing 0.2 ml BCG were offered to three white-tailed deer during January 2012 on top of the maintenance feed in the previously described 19.5 m×10 m pens. These three deer were not included in the bait consumption analysis described earlier.

Results

The physical stability tests showed that environmental temperature alone did not significantly impact bait stability. All baits remained within 10 % of their initial thickness when only environmental temperature varied (Fig. 2). Exposure to water

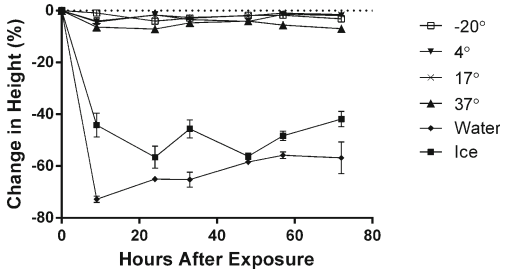

Fig. 2 Physical stability of baits when exposed to various temperatures (−20, 4, 17, and 37 °C) and moisture conditions (i.e., water and ice). Decreased stability is indicated by greater reduction in thickness in response to application of 300-g weight for 10 min. Data are presented as mean +/− SEM percent change in height

or crushed ice, in contrast, did cause a significant decrease in bait thickness by at least 40 %.

Storage temperature (i.e., −80, −20, or 4 °C) and days at respective various temperatures (i.e., 3, 17, and 31 days) did not cause a significant ($p > 0.05$) decrease in BCG viability. At −80 °C, BCG remained viable at 7.73×10^6 CFU 31 days after inoculation with 1×10^8 CFU of BCG compared to 5.2×10^6 CFU at −20 °C and 1.32×10^6 CFU at 4 °C, indicating that −80 °C may be an optimal storage temperature. The mean CFU slightly decreased by day 31 in baits stored at 4 °C from 3.11×10^6 CFU on day 3 to 1.32×10^6 CFU. Although the change by day 31 was not statistically significant from days 3 and 17 ($p > 0.05$), it illustrates that viability is less certain if baits are exposed to temperatures above 4 °C for over 31 days.

Near complete consumption of baits was observed repeatedly over 10 days when evaluating sham bait palatability. Forty-eight of the 50 trials were completely consumed within 3 h. Review of images captured by the digital game camera showed that >62 % of the time, all baits were consumed immediately by a single deer. This demonstrates that placing baits in a single location (e.g., a bait station) could cause overconsumption of the vaccine by certain deer and inadequate vaccine coverage of the target population. Inoculating baits with BCG did not have a negative impact on palatability as all the BCG-containing baits were readily consumed.

Discussion

Efficient uptake of baits in this study demonstrates that a molasses-based bait formulation is palatable to white-tailed deer. However, the current study used captive deer, habituated to regular feeding. Field studies will be necessary to determine the attractiveness of such bait to free-ranging deer. As apple flavor, apple odor, and corn have also been used as effective attractants for free-ranging white-tailed deer, it may be advantageous to use a combination of attractants for optimum attraction.

Results of the physical stability test determined the bait matrix to be stable in dry conditions but unstable when in contact with ice and water. This suggests acceptable bait stability during winter with constant temperatures below freezing; however, if ambient temperatures result in melting snow and ice, bait stability will be compromised. It may prove advantageous to use a device such as the "four-poster" feeder, or other mechanism to shield baits from ice and water (Slate et al. 2009).

The current study also demonstrates that this molasses-based bait may be a feasible means of oral delivery of BCG to white-tailed deer. In general, virulent *M. bovis* can survive for days, weeks, or even months on feedstuffs in cooler, shaded areas (Fine et al. 2011). However, current BCG viability results suggest decreasing viability if baits are exposed to temperatures above 4 °C for over 31 days. A certain degree of survivability is required of any live vaccine delivered via oral bait. The vaccine must remain viable for a reasonable amount of time, such as the interval from deployment to consumption. However, greatly prolonged survivability may contaminate the environment and increase the likelihood of nontarget species (including cattle) exposure to vaccine. While deployment of a BCG-containing bait in winter decreases contact with nontarget species that are not active during winter, some nontarget exposure is likely to occur. Vaccine safety in nontarget species is a legitimate concern though the risk transmission of BCG to cattle was shown to be low (Nol et al. 2013). Importantly, *M. bovis* BCG is one of the oldest vaccines still in use and has been extensively used in animal models of human tuberculosis (e.g., mice, guinea pigs, and rabbits) with no deleterious effects (Clark et al. 2010, Baldwin et al. 1998).

Prolonged survivability in the environment as well as prolonged survivability within the deer represent public health concerns, as deer (venison) is often consumed by hunters. Examination of persistence in deer after oral or parenteral BCG vaccination demonstrated persistence in lymphoid tissues (i.e., lymph nodes) for extended periods, up to 12 months. However, BCG was never isolated from tissues commonly consumed by humans (Palmer et al. 2012, Wilkins et al. 2003). BCG is considered avirulent in immunocompetent hosts; however, human exposure to BCG may induce false positive tuberculin skin test results. False positive results are undesirable as tuberculin skin testing is the primary means by which public health officials monitor for human tuberculosis.

This study also revealed the potential for overconsumption of baits by certain deer, leading to variability in dose and inadequate vaccine coverage within the target population. Deer consuming numerous baits not only decreases the remaining number of baits and negatively impacts vaccine coverage but also results in ingestion of a greater than desired dose of BCG by eager deer. According to Nol et al. (2008), an oral dose of 10^9 CFUs provides protection to experimentally infected deer. It is not clear what effect high BCG dosages have on vaccine efficacy. The minimal oral dose of BCG required to provide protection is also unclear

Ultimately, such oral BCG vaccination of deer may provide a useful tool in the tuberculosis eradication effort of the Michigan Department of Natural Resources (MDNR). However, future studies are needed to determine how soon after vaccination BCG will provide protective immunity, as well as the duration of immunity. Thom et al. (2012) determined, in cattle, that BCG provided protection against *M. bovis* at 1-year post vaccination but not 2 years, indicating that repeated vaccination might be required. When the timeline of protective immunity is known, the most effective means of vaccine deployment can be determined. Piles of supplemental feed, provided by humans, are an important factor in deer-to-deer transmission of *M. bovis* (Palmer et al. 2004). Disease is transferred via exchange of infectious excretions deposited on shared feed. Therefore, it will be important to design a bait delivery method that does not unnaturally congregate deer, unintentionally creating an environment for enhanced disease transmission.

The baits developed in this study represent a plausible option for delivery of BCG to white-tailed deer. Distribution of baits during winter months will enhance BCG survival and limit nontarget species exposure. Nevertheless, field evaluation of bait consumption by nontarget species should be conducted to determine any deleterious effects should unintended ingestion occurs. Additionally, bait palatability studies and survivability of BCG within baits in the field during the targeted delivery season are needed to fully evaluate the potential for use of these vaccine delivery baits to deliver BCG to white-tailed deer in the TB core area of Michigan.

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