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Field evaluation of a *Mycoplasma bovis* bacterin in young dairy calves

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ABSTRACT

Mycoplasma bovis is an important cause of pneumonia, otitis media and arthritis in young dairy calves, and there is a critical need for improved preventative strategies for this pathogen. We conducted a randomized, placebo-controlled, double-blinded field trial to determine the efficacy of a commercial *M. bovis* vaccine for the prevention of *M. bovis*-associated disease in calves. Calves ($n = 373$) on 3 Florida dairies with a history of *M. bovis* infection received an *M. bovis* bacterin or a placebo, administered subcutaneously at 3, 14 and 35 days of age. One of the herds did not experience *M. bovis*-associated disease; for calves in the remaining 2 herds, the incidence risk for respiratory disease, otitis media and arthritis from 3 to 90 days of age was 0.64, 0.28 and 0.02, respectively. Vaccination had no effect on the age at first treatment for *M. bovis*-associated disease, incidence of respiratory disease, mortality, weight gain, or nasal colonization with *M. bovis* in the first 90 days of life. In one herd, vaccination was associated with an increased risk of otitis media. There was no association between *M. bovis*-specific serum antibody titers and morbidity in vaccinated calves. Under the field conditions in this study, this vaccine was not efficacious for the prevention of *M. bovis*-associated disease in young dairy calves.

1. Introduction

Mycoplasma bovis is a significant world-wide pathogen of adult dairy cows as well as intensively reared beef and dairy calves [1–8]. In the past decade, *M. bovis* has emerged as an increasingly important cause of respiratory disease, otitis media and arthritis in young calves less than 3 months of age [1,2,6,7,9]. Clinical disease caused by *M. bovis* tends to be chronic, debilitating and unresponsive to antimicrobial therapy [8–14]. Disease outbreaks with high morbidity rates occur [1,2,10,15,16] and can be economically devastating for the affected farm. The costs of infection are primarily associated with intensive treatment of affected calves coupled with culling of animals that are unresponsive to therapy [6]. *M. bovis*-associated disease is also important from an animal welfare perspective as it often results in calves that are subject to severe, chronic illness for which the producer or veterinarian can provide only limited relief. There is therefore a critical need to develop improved preventative and treatment strategies for *M. bovis*-associated disease.

Currently, control of *M. bovis* infection in calves focuses on removal of identified or potential risk factors. Colonization of the upper respiratory tract of calves with *M. bovis* often occurs within the first few weeks of life [1,17] as a result of feeding of milk from cows infected with *M. bovis* or, probably, by direct or indirect transmission from other calves shedding *M. bovis* in nasal secretions. Removal of infected milk from the diet by pasteurization or feeding of milk replacer has been successfully applied to reduce infection [1,15,16,18,19]. Breaks in pasteurization have been associated with subsequent infection outbreaks. Management practices to reduce stocking density and improve ventilation are examples of changes that can reduce undifferentiated respiratory disease in housed calves and have been recommended for *M. bovis* control [20–22]. Similarly, control of other pathogens that are involved in the bovine respiratory disease complex is likely to reduce *M. bovis* infections. Management techniques that improve general immune function, such as improving nutritional status and minimizing environmental stress, have also been suggested as beneficial [21,22].

Vaccination is a potential strategy to control *M. bovis* infection, but efforts to develop efficacious vaccines against *M. bovis* for use in young calves have been problematic. Vaccines against *M. bovis* have afforded some protection from respiratory disease in European field trials [23–25]. Other vaccines have been efficacious against respiratory disease [26,27] and arthritis [26,28,29] in experimental challenge studies. However, in some cases vaccination against *M. bovis* has significantly exacerbated clinical disease [30,31]. In addition, most experimental challenge studies have been performed in

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calves that are substantially older than the age at which natural colonization with *M. bovis* is typically first observed. Young calves are often colonized by *M. bovis* within the first few weeks of life [1,2,15,16,32], during which time the immune system is undergoing rapid changes associated with maturation [33,34]. Therefore, age-specific features of the immune system are likely to be important in determining the susceptibility of the young calf to mycoplasmal disease and the efficacy of particular vaccines.

There are several bacterin-based vaccines against *M. bovis* that are currently marketed in the U.S., as well as a number of companies that manufacture autogenous *M. bovis* bacterins. However, no commercial vaccines are licensed for use in the very young dairy calf, and, to the best of the author's knowledge, no independent studies have been published on their efficacy. The paucity of studies that critically evaluate currently marketed *M. bovis* vaccines and autogenous bacterins in well-designed, independent efficacy studies in an appropriate age group is a major gap in understanding the potential of currently available vaccines as a management strategy to control *M. bovis* infections in young calves. In part to address this gap, we conducted a field trial using a commercial *M. bovis* bacterin that was approved for use in feeder and stocker calves. The objective of this field trial was to determine the efficacy of this commercially produced *M. bovis* bacterin for the prevention of *M. bovis*-associated disease (respiratory disease, otitis media, arthritis) and mortality in dairy calves up to 90 days of age. Additional objectives were to compare vaccinated and placebo-treated calves with respect to (1) weight gain from birth to 90 days of age, (2) rates of nasal colonization by *M. bovis*, and (3) *M. bovis*-specific serum immunoglobulin (Ig) concentrations.

2. Materials and methods

2.1. Study populations

We studied 373 Holstein heifers in three Florida herds using a randomized field trial design. The reference population for this study was heifer calves in Florida dairy herds with endemic *M. bovis* infection. The study unit was a Holstein heifer calf clustered in one of the three herds in north-central Florida. Herds were selected based on their willingness to participate and on a history of mycoplasma-associated disease in calves. According to calf health records, at least 15% of calves were treated for respiratory disease, otitis media and/or arthritis during each of the 2 years preceding the study. Calves were enrolled from March to December, 2002.

Herd A had approximately 500 lactating cows. Calves were bedded on sand in individual hutches placed approximately 1 m apart in an open-sided barn with mechanical ventilation. Calves were fed unpasteurized bulk tank milk. Calves received a modified live virus (MLV) intranasal vaccine against parainfluenza virus type 3 (PI_3) virus and infectious bovine rhinotracheitis virus (IBR) in the first week of life. An intramuscular MLV vaccine against IBR, PI_3, bovine respiratory syncytial virus (BRSV) and bovine viral diarrhea virus (BVDV) types 1 and 2 was administered at 2, 6 and 8 weeks of age. A 7-way clostridial vaccine was administered at 2 and 6 weeks of age. Calves were weaned at approximately 6 weeks of age and turned out into group pens at approximately 8 weeks of age.

Herd B had approximately 750 lactating cows. The majority of calves were housed in individual elevated metal crates in a concrete-floored open-sided barn with mechanical ventilation. A small proportion of calves were housed in individual hutches on grass. Calves housed in metal crates had nose-to-nose contact with neighboring calves. The feeding protocol varied during the study period and included milk replacer and unpasteurized or pasteurized waste milk. The vaccination protocol was similar to that described for Herd A. Calves were weaned at 6–8 weeks of age and turned out into group pens at 8–10 weeks of age.

Herd C had approximately 1000 lactating cows. Calves were housed on grass in individual hutches placed at least 1 m apart. Calves were primarily fed pasteurized waste milk, supplemented with milk replacer when necessary. Several failures of pasteurization were documented during the study period through culture of milk following pasteurization. Calves received an oral bolus containing antibodies against bovine coronavirus and *Escherichia coli* at the time of colostrum feeding (First Defense, Portland, ME). The vaccination protocol for MLV intranasal PI_3/IBR and clostridial vaccines was similar to that described for Herd A except that the intramuscular MLV vaccine against PI_3, IBR, BVDV types 1 and 2, and BRSV was administered at 4 and 8 weeks of age. Calves were weaned at 6–8 weeks of age and turned out into group pens at 8–10 weeks of age.

2.2. Study design and sample collection

All Holstein heifer calves that were born during the study period and were considered healthy by the producer at 3 days of age were enrolled in the study. Calves were assigned to one of the two groups based on ear-tag numbers, with odd numbers assigned to one group and even numbers to the other group. Assignment of odd and even numbers to groups was decided on a per farm basis by a coin flip. One group received a 1 ml dose of a killed, single strain *M. bovis* bacterin in proprietary oil-based adjuvant that had a conditional license for the prevention of respiratory disease in U.S. feeder and stocker calves at the time of the study (Texas Vet. Labs, Inc., San Angelo, TX), while the other group received a placebo (all vaccine components except antigen; control group). Vaccine or placebo was administered subcutaneously in the neck at 3 and 14 days of age. A 2 ml dose was administered at 35 days of age. The second or third doses were not administered to calves that were sick; however, if the calf recovered within 5 days the dose was administered 1 week after the due date. Calves that failed to recover within 5 days remained in the study but missed that dose; missed doses were recorded. The bacterin and placebo were prepared and blinded by the vaccine manufacturer, and the investigators and farm personnel were blinded throughout data collection and analysis. Data recorded for each calf included date of birth, ear-tag number, group allocation, dates of vaccine/placebo administration, any missed doses of vaccine/placebo, and date of weaning. The dates of administration of any preventative treatments or other vaccines were recorded for each calf.

The primary outcomes of interest were treatment for respiratory disease, otitis media, and arthritis as well as mortality attributed to these diseases. Calves were followed from 3 days until 90 days of age, during which time all treatment for clinical disease was recorded by farm personnel using standardized case definitions. Farm personnel were trained by the investigators and followed a standardized protocol for health assessment that was prepared by a veterinarian. Respiratory disease was defined as fever (rectal temperature $\geq$103.5 °F) plus increased respiratory rate or effort and/or coughing and/or nasal discharge; otitis media/interna was defined as ear droop and/or evidence of ear pain (head shaking, scratching or rubbing ear); arthritis was defined as lameness with painful swelling of any joint. Sick calves were treated as per normal farm protocols. For each clinically ill calf, farm personnel recorded the type and dose of antimicrobial, the date(s) of treatment, and the reason for treatment. Whenever a calf died, farm personnel recorded the cause of death if this was obvious. In most cases, cause of death was verified by field necropsy performed by the investigators. Study personnel visited each of the dairies at least once a week to collect calf health data, monitor compliance, and collect samples. Because passive transfer of colostral immunoglobulins can

influence the immune response to vaccination or to infectious agents, blood was collected from all calves between 2 and 9 days of age for the measurement of total serum protein concentration.

A subset of 100 calves from Herds A ($n = 40$) and B ($n = 60$) was studied more intensively. These calves were weighed at birth and approximately 90 days of age. Weight gain from birth to 90 days was expressed in kg/day. Nasal swabs and blood samples were collected weekly until 8 weeks of age and then at 90 days of age. Prior to collecting nasal swabs, gross debris was wiped from the external nares using sterile gauze. A sterile rayon-tipped swab with a polyurethane plastic shaft (BBL™ CultureSwab™ Liquid Stuart Medium, BD, Franklin Lakes, NJ) was inserted into the ventral nasal meatus to a depth of approximately 4 in. Swabs were cultured to detect nasal colonization with *M. bovis*. Serum was analyzed for *M. bovis*-specific IgA, IgM, IgG_1 and IgG_2 by enzyme-linked immunosorbent assay (ELISA).

2.3. Culture and identification of M. bovis

Swabs were kept on ice during transport and were processed within 6 h of collection. Each swab was used to streak the surface of modified Frey's agar. All mycoplasma cultures were performed in modified Frey's broth and agar medium containing 2.25% (wt./vol.) Mycoplasma broth base (Frey) (BD Diagnostic Systems, Sparks, MD), 0.02% (wt./vol.) DNA from herring sperm, 20% (vol./vol.) horse serum, 10% (vol./vol.) fresh yeast extract, 0.5% (wt./vol.) glucose, and supplemented with 100,000 U/l each of penicillin G and polymixin B and 65 mg/l of cefoperazone, with the final pH adjusted to 7.6–7.8. Plates were incubated at 37 °C in 5% CO_2 and examined at 2, 4, 7 and 10 days for mycoplasmal growth. Colonies with typical *M. bovis* morphology were plugged into broth, incubated at 37 °C for 48 h and stored at −80 °C until they could be confirmed as *M. bovis* by polymerase chain reaction (PCR).

To prepare samples for PCR, 500 μl of broth culture was thawed at room temperature then pelleted by centrifugation at 14,000 rpm at 4 °C for 1 h. The pellet was resuspended in 20 μl of lysis buffer (100 mM tris [hydroxymethyl] aminomethane, pH 7.5 with 0.05% [vol./vol.] Tween 20 and 6.5 mM dithiothreitol). Samples were incubated at 99 °C for 20 min then cooled to 20 °C. 5 μl of clarified sample was used as the DNA template in the PCR. As a positive control, broth was inoculated with the *M. bovis* type strain PG45 and processed with nasal isolates. Sterile water was used as a negative control template. *M. bovis* was identified by PCR of the housekeeping gene *uvrC*, as previously described [35]. PCR products were analyzed by electrophoresis at 65 V for 1 h in 1.5% agarose gels and visualized by staining with ethidium bromide.

2.4. Serology

Blood samples were allowed to clot after collection, and then serum was harvested by centrifugation and stored at −80 °C. Whole-cell lysate antigen [36] was prepared from a 1 l culture of *M. bovis* type strain PG45 grown at 37 °C in modified Frey's broth. The protein concentration was determined using a colorimetric assay (Bio-Rad, Hercules, CA) and adjusted to 100 μg/ml. The antigen was stored in aliquots at −80 °C and thawed at room temperature when required. The ELISA procedure was optimized using standard methodology. Microtiter plates (Maxisorb F96, Nunc, Kamstrup, Denmark) were coated with 20 μg per well of antigen in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.02% (wt./vol.) NaN_3 (PBS/A), and incubated overnight at 4 °C. Plates were then washed three times with PBS/A containing 0.05% (vol./vol.) Tween 20 (PBS/T) using an automated plate washer (ELx405 Auto Plate Washer, BioTek Instruments, Inc., Winooski, VT), blocked with 300 μl per well of blocking buffer (PBS/T containing 1% [wt./vol.] egg albumin), and stored at 4 °C for a minimum of 24 h or until needed. Sera were diluted (1:100 for IgG_1 assay; 1:50 for IgM and IgG_2 assays; 1:25 for IgA assay) in blocking buffer and 50 μl of the diluted serum was added to duplicate wells; plates were incubated at room temperature for 1 h. Plates were washed as described above and 50 μl of goat anti-bovine isotype conjugated to alkaline phosphatase (Bethyl Laboratories Inc., Montgomery, TX) and diluted to 1:1000 in blocking buffer was added to each well. Plates were incubated at room temperature for 2 h and then washed as described above. 100 μl of 0.1% (wt./vol.) p-nitrophenol phosphate was added to each well and plates were incubated in the dark at room temperature for 1 h. The optical density (OD) in each well was read at a wavelength of 405 nm using an automated plate reader (ELx808 Ultra Microplate Reader, BioTek Instruments, Inc., Winooski, VT). For each microtiter plate, the blank was the mean value for two wells coated with antigen and incubated with the conjugated secondary antibody and substrate only. The blank OD value was subtracted from each sample well, and mean values for each pair of duplicate tests calculated.

A pool of sera from 20 calves with naturally occurring mycoplasmal disease and high *M. bovis*-specific titers was included on each plate as a positive control; the negative control was a pool of serum collected from the same 20 calves prior to ingestion of their first colostrum meal. The cutoff for a positive result was the average OD value (minus the blank) for the negative control sera plus two standard deviations, established over ten assay runs. All ELISA data was reported as the average OD value at the standard serum dilution for each isotype. Within-batch and between-batch assay variabilities were assessed by using the Youden plot graphic method [37]. The ELISA values obtained for the lowest, middle and highest dilution of the control serum included on each plate were used to establish target values and control limits to be used for monitoring the consistency of the assay (ten batches). The values obtained at the beginning of a series of assays were plotted against the values obtained for the same standards at the end of the series. If values for the pooled sera deviated more than 10% from target values, the assay was repeated.

2.5. Field necropsy

A standard field necropsy was performed by a study veterinarian on most calves that died or had to be euthanized. Selected calves were not necropsied when a cause of death was obvious and there had been no clinical evidence of *M. bovis* infection (e.g. a leg fracture that necessitated euthanasia). Calves were examined to determine the cause of death and specifically determine the involvement of *M. bovis*-associated pathology. Cases were defined as *M. bovis*-associated mortality when there had been a history of respiratory disease, otitis media, and/or arthritis, where *M. bovis* (with or without other pathogens) was isolated from sites of pathology, and where the observed pathology was consistent with mycoplasmal disease. All necropsies included collection of swabs of the palatine tonsils, tympanic bullae and primary bronchi for mycoplasmal culture. Additionally, if the animal had previously been diagnosed with respiratory disease, arthritis or otitis media, or if any macroscopic lung pathology was observed, appropriate samples were collected from the lesion site(s) to determine the involvement of *M. bovis* as well as other viral and bacterial respiratory pathogens. Further samples were collected when deemed necessary to determine the cause of death by the veterinarian performing the necropsy. All swabs and fresh tissue samples were transported on ice to the laboratory as soon as possible and were processed within 24 h after collection. When tissue samples for fixation were collected, they were placed into containers of 10% buffered formalin and submitted to the Diagnostic Pathology Service, College of Veterinary Medicine, University of Florida. Samples then were embedded in paraffin and sections (5 μm) stained with hematoxylin and eosin.

Histopathology was read by diagnostic pathologists without knowledge of experimental treatment groups. In addition to culture for mycoplasmas (described under nasal swabs, above), swabs for aerobic microbiological culture were processed and isolates identified using routine clinical bacteriological methods. These methods were focused on identifying bacterial pathogens of the respiratory tract other than mycoplasmas, particularly *Arcanobacterium pyogenes*, *Histophilus somni*, *Mannheimia haemolytica* and *Pasteurella multocida* as well as pathogens that may cause septicemia and associated sequelae in young calves. Additional diagnostic testing was performed as requested by the veterinarian who conducted the necropsy based on the presumptive diagnosis and any macroscopic pathology. Samples were submitted to the Florida State Diagnostic Laboratory (Kissimmee, FL) for detection of bovine respiratory viruses when indicated.

2.6. Sample size and statistical methods

Morbidity due to *M. bovis* was the major outcome of interest and was therefore used to calculate sample size. At the time the study was initiated, health records indicated that the incidence of respiratory disease, otitis media and/or arthritis in the study herds was at least 15%. We hypothesized that a reduction in incidence to 5% would be biologically and economically significant. Using these values together with 95% confidence and 80% power, and taking into consideration an attrition rate of approximately 10%, the calculated sample size was 180 calves per group. For the secondary outcomes of interest from a subset of calves in Herds A and B, we hypothesized that a reduction in the nasal colonization rate from 50% to 20% would be biologically significant. Using these values together with the parameters outlined above, the calculated sample size was 50 calves per group.

Calves were excluded from analyses if clinical signs referable to other organ systems occurred concurrently with respiratory disease, otitis media or arthritis, with the exception of diarrhea without fever of less than 7 days duration. Incidence risk rates were calculated as the number of calves treated for the disease of interest divided by the initial number of calves less half the calves that died during the study for reasons other than the disease of interest. Categorical outcome variables (incidence of clinical disease [%], incidence of mortality [%], incidence of missed dose of vaccine/placebo [%], cumulative risk of nasal colonization [%]), were compared among groups using chi-square tests; data were analyzed for effects of herd and passive transfer status by Stratified Mantel–Haenszel analysis. When the expected count in any cell was less than 5, Fisher's exact test was used. Quantitative outcome variables (post-colostral total serum protein concentrations [g/dl], age at first treatment for clinical disease [days], average daily weight gain from birth to 90 days of age [kg/day]) were compared among groups using independent sample *t*-tests, and ELISA data were analyzed using repeated measures ANOVA. The relationships between average daily weight gain and serological data (post-colostral ELISA data, week 12 ELISA data) were examined using Pearson correlation analyses. A P value of < 0.05 was considered significant. All analyses were performed using commercial statistical software (SPSS 12.0, SPSS Inc., Chicago, IL).

3. Results

3.1. Enrollment and incidence risk for M. bovis-associated disease

Three hundred and twenty-eight calves from Herds B and C (166 and 162 calves, respectively) were enrolled and were eligible for inclusion in the study (Table 1). Despite a history of *M. bovis* infection, Herd A did not experience any *M. bovis*-associated disease during the study and therefore is excluded from some analyses, but data from this herd are included where relevant. The incidence risk for clinical respiratory disease, otitis media, and arthritis was assessed from 3 to 90 days of age (Table 2). *M. bovis*-associated respiratory disease and otitis media were major contributors to calf disease in Herds B and C. Eight cases of arthritis were observed in Herd B, and none were observed in Herd C. Herd A had a much lower overall mortality risk (0.02) than did Herds B (0.13) and C (0.10); *M. bovis*-associated mortality in Herd B accounted for the majority of the mortality risk (0.10 vs. 0.13 overall).

Table 1
Summary of calves enrolled in vaccine field efficacy study.

Herd	Vaccinated	Control	Exclusions[a]	Total
A	21	20	0	41
B	81	85	2	168
C	82	80	2	164
All herds	184	185	4	373

[a] In Herd B, one calf was excluded because of concurrent disease and one calf was excluded because a dose of vaccine/placebo was inadvertently missed; in Herd C, one calf was excluded because of concurrent disease and one calf was excluded because it received a dose of vaccine/placebo for the wrong group.

Table 2
Incidence risk rate[a] for *Mycoplasma bovis*-associated disease and mortality between 3 and 90 days of age in calves in the three study herds.

	Herd A	Herd B	Herd C
Disease			
All *M. bovis*-associated	0.00	0.55	0.74
Otitis media	0.00	0.22	0.35
Respiratory disease	0.00	0.48	0.69
Arthritis	0.00	0.04	0.00
Other	0.07	0.15	0.19
Mortality			
M. bovis-associated	0.00	0.10	0.03
All causes	0.02	0.13	0.10

[a] Incidence risk rate: no. of calves that developed the disease of interest/initial no. of calves − 1/2 no. of calves died for reasons other than disease of interest.

3.2. Baseline data

Vaccinated and control groups had equivalent levels of post-colostral total serum protein. A small percentage of calves did not receive their second vaccine/placebo due to illness, and this was not different between groups. Although there were no significant differences for calves that missed the third vaccine/placebo when data from Herds B and C were combined (7% [12/163] vaccinate vs. 5% [8/165] control), no control calves in Herd B missed the third placebo as compared with 9% (7/81) of vaccinated calves that missed the third vaccine ($P = 0.005$).

3.3. Effect of vaccination on M. bovis-associated morbidity and weight gain

Vaccination was not efficacious in limiting *M. bovis*-associated morbidity in the young dairy calves in this field trial. Vaccination did not influence the age of first treatment for either otitis media or respiratory disease. The overall age for first treatment of otitis media was 32 ± 13 days for vaccinated calves and 31 ± 13 days for control animals. Calves in Herd C were treated for otitis at 27 ± 10 days (vaccinated) and 24 ± 9 days (control), whereas treatment was at a later age in Herd B (37 ± 16 days and 37 ± 17 days for vaccinated and control calves, respectively). A similar pattern was observed for respiratory disease, with no difference in the overall age at first treatment between vaccinated and control calves (24 ± 15 days, vaccinated; 25 ± 16 days, control). As for otitis media, calves in Herd

Table 3
Morbidity due to respiratory disease and otitis media in vaccinated and control calves.

	Vaccinated		Control		*P*
	%	No.	%	No.	
Herd B					
Respiratory only	22	18/81	31	26/85	0.222
All respiratory	47	38/81	42	36/85	0.555
Otitis media only	5	4/81	1	1/85	0.202
All otitis media	30	24/81	12	10/85	0.004
Arthritis[a]	3	2/81	5	4/85	0.682
All *Mb*AD	54	44/81	47	40/85	0.350
Herd C					
Respiratory only	43	35/82	31	25/80	0.132
All respiratory	67	55/82	64	51/80	0.657
Otitis media only	4	3/82	5	4/80	0.718
All otitis media	28	23/82	38	30/80	0.200
Arthritis	0	0/82	0	0/80	–
All *Mb*AD	71	58/82	69	55/80	0.784
Herds B and C[b]					
Respiratory only	33	53/163	31	51/165	0.755
All respiratory	57	93/163	53	87/165	0.431
Otitis media only	4	7/163	3	5/165	–
All otitis media	29	47/163	24	40/165	–
Arthritis	1	2/163	2	4/165	0.685
All *Mb*AD	63	102/163	58	95/165	–

Results are expressed as the number of calves with disease/total number of calves in that group. Herd A calves were excluded as no morbidity was observed in these animals. "All respiratory" includes calves that were treated for respiratory disease alone or for respiratory disease in conjunction with either otitis media or arthritis. "All otitis media" includes calves that were treated for otitis media alone or for otitis media in conjunction with either respiratory disease or arthritis. *Mb*AD = *Mycoplasma bovis*-associated disease.

[a] One control calf with arthritis also had respiratory disease.

[b] Combined (Herds B and C) analyses were not performed for data that included otitis media because of the interaction between vaccination and Herd for this outcome.

C required earlier treatment for respiratory disease (20 ± 17 days, vaccinated; 21 ± 17 days, control) than did calves in Herd B (30 ± 13 days, vaccinated; 33 ± 14 days, control).

Vaccination did not reduce the overall *M. bovis*-associated morbidity or the morbidity specifically associated with otitis media or respiratory disease. In fact, vaccination was associated with increased incidence of otitis media ($P = 0.004$) in Herd B, but no differences in the incidence of otitis media between groups were observed in Herd C (Table 3).

For Herds A and B, weight gain was monitored from birth to 90 days of age; no significant difference was observed in average daily gain between groups in Herd A. Similarly, no significant difference was observed in average daily gain between vaccinated (0.48 ± 0.18 kg/day, $n = 27$) and control (0.54 ± 0.11 kg/day, $n = 29$) calves in Herd B, where endemic *M. bovis* disease was present.

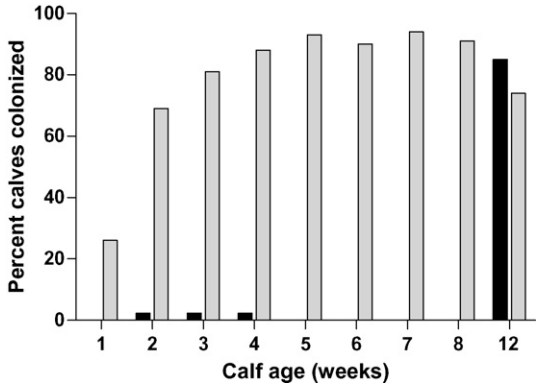

Fig. 1. Temporal pattern of nasal colonization of calves by *Mycoplasma bovis* in herds without mycoplasmal disease (Herd A; black bars) or with mycoplasmal disease (Herd B; gray bars). Results are expressed as the percent of calves sampled from which *M. bovis* was isolated from the nares at each time point.

3.4. Effect of vaccination on *M. bovis*-associated mortality

Neither overall mortality rates (vaccinated, 10% [17/163]; control, 13% [21/165]) nor *M. bovis*-associated mortality rates (vaccinated, 6% [10/163]; control, 7% [11/165]) differed significantly between vaccinated and control calves. In Herd B, *M. bovis*-associated mortality contributed to 82% (9/11) and 70% (7/10) of mortality events in vaccinated and control calves, respectively. Although *M. bovis*-associated mortality also occurred in Herd C, it was not the primary cause of calf mortality: only 17% (1/6) of mortalities in vaccinated and 36% (4/11) of mortalities in control calves in Herd C were attributable to *M. bovis*.

3.5. Nasal colonization

Nasal colonization was not affected by vaccination; in Herd B where endemic *M. bovis* disease was present, the mean percentage (±SD) of calves with *M. bovis*-positive nasal swabs at each sampling time was 81.4 ± 24.4% for vaccinated calves and 75.8 ± 20.0% in control calves. The average number of sampling times that *M. bovis* was recovered from each calf was also not associated with vaccination (data not shown). Irrespective of vaccination status, however, the temporal pattern of colonization (Fig. 1) observed in calves from Herd A (no mycoplasmal disease) was quite different from that observed in calves in Herd B (significant mycoplasmal disease). Calves in Herd A had minimal to no nasal colonization with *M. bovis* during the pre-weaning period. Calves in Herd A were moved out of individual hutches into outdoor housing in group lots after the 8-week samples were collected; at the next sampling period (12 weeks of age), the level of nasal colonization was similar to that in the herd that experienced *M. bovis*-associated disease. In Herd B, calves had *M. bovis* present in nasal secretions as early as 1 week of age, and by 3 weeks of age over 70% of calves were colonized in the upper respiratory tract. This level of colonization was maintained throughout the sampling period.

3.6. Antibody response

The serum antibody subclass response in a subset of calves in Herds A and B was assessed by ELISA (Fig. 2). No significant differences between vaccinated and control calves were found in either Herd A or B for IgA, IgM, or IgG_2. However, a serum IgG_1 response to vaccination was detected. Significant differences ($P < 0.05$) between vaccinated and control groups were first evident at 7 weeks of age in Herd A (no endemic disease) and at 12 weeks of age in Herd B (endemic disease). We then assessed if there was an association between immunoglobulin subclass response and outcomes in calves from Herd B. There was no association of any immunoglobulin subclass response with morbidity or nasal colonization rate (data not shown). The *M. bovis*-specific serum IgG_1 titers at 12 weeks of age were negatively correlated with average daily gain for control calves ($r = -0.644$, $r^2 = 0.415$, $P < 0.001$), but not for vaccinated calves ($r = -0.218$ $r^2 = 0.05$, $P = 0.284$).

Interestingly, there was no significant association between post-colostral total serum protein concentrations and the incidence or duration of treatment for respiratory disease or otitis media in Herds B and C (data not shown). Similarly, the incidence of *M. bovis*-specific calf mortality in Herds B and C was not associated with post-colostral total serum protein concentrations (data not shown). There was also no association between post-colostral *M. bovis*-specific IgA, IgM, IgG_1 or IgG_2 serum titers and either

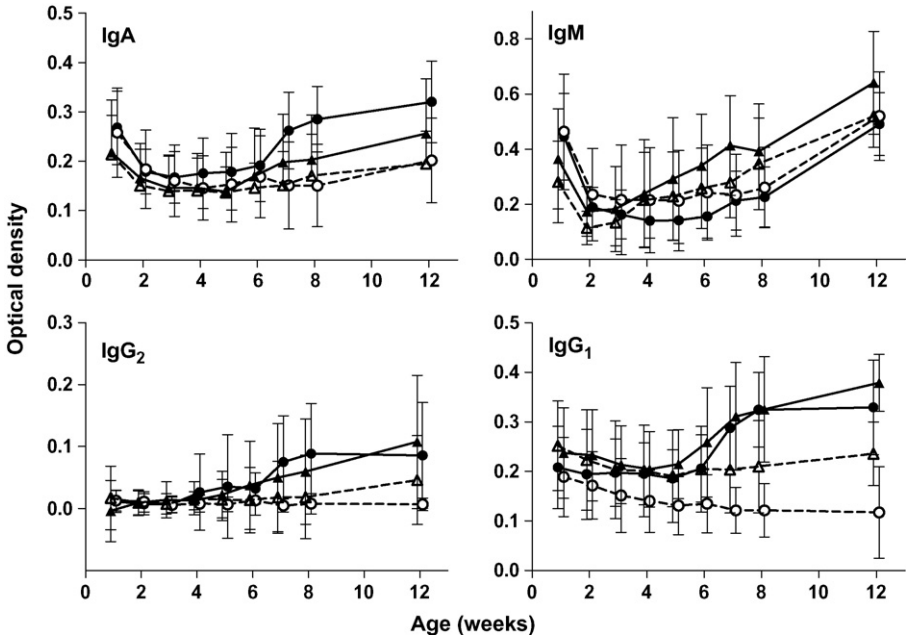

Fig. 2. Specific immunoglobulin subclass response to *Mycoplasma bovis* in vaccinated (solid line) and control (dotted line) calves in Herd A (●,○) and Herd B (▲,△). No significant differences were observed between vaccinated and control calves in the IgA, IgM, or IgG_2 subclasses. Vaccinated calves in Herd A at 7, 8 and 12 weeks of age and in Herd B at 12 weeks of age had significantly higher levels ($P < 0.05$) of IgG_1 than did control calves at those time points. Results are presented as mean optical density ± SD.

morbidity or mortality for the intensively studied subset of calves ($n = 60$) in Herd B (data not shown).

4. Discussion

The commercial *M. bovis* bacterin tested in this trial was not efficacious in the prevention of either *M. bovis*-associated respiratory disease or otitis media in pre-weaned calves in two Florida herds with endemic *M. bovis* disease. The response to vaccination was herd-dependent, and a higher rate of otitis media was associated with vaccination in one herd. Our findings are not unique; although other investigators have reported some protection from *M. bovis*-associated disease by vaccination of older calves [23,24,26,27], adverse outcomes following vaccination against *M. bovis* have also been reported [30,31,38].

Protection from mycoplasmal respiratory disease by subcutaneous vaccination of calves with killed whole cell bacterins has been reported [23,24,26,27]. In a study of an apparently efficacious vaccine in young calves, Nicholas et al. [26] vaccinated 3-week-old dairy calves with a single dose of inactivated saponin-adjuvanated bacterin. Calves received an aerosol challenge with live *M. bovis* 3 weeks after vaccination. Vaccinated calves had fewer numbers of *M. bovis* at colonized sites, fewer body sites colonized by *M. bovis*, and reduced severity and incidence of clinical disease and lesions as compared to control calves. There was also a significant decrease in body weight gain in control calves compared with vaccinates. Additionally, no vaccinated calves and two of seven control calves developed arthritis. Vaccinated calves produced a strong IgG response prior to challenge, but IgG subtypes were not reported. No adverse events associated with vaccination were reported.

A killed vaccine against four bovine respiratory pathogens (BRSV, PI_3, *M. bovis*, and *M. dispar*) was evaluated for protection against naturally occurring respiratory disease in beef calves [23,24]. Calves were vaccinated subcutaneously and received two boosters at 3-week intervals. In one study [24], three groups of beef calves aged 12, 7 and 3 weeks at the time of first vaccination were used, and calves were followed for 6 months. Respiratory disease occurred in a significantly higher ($P < 0.05$) proportion of the control calves (27%) compared with the vaccinates (16.3%). In a second study [23] using the same vaccination protocol, *M. bovis* and BRSV were implicated in outbreaks of respiratory disease during the trial period. Morbidity due to respiratory disease was significantly reduced in vaccinated calves (25%) compared with controls (32%), and mortality in the vaccinated group was similarly reduced (2% and 9% for vaccinates and controls, respectively). No adverse effects of vaccination were noted.

There are a number of key differences between the studies reported above and our study that may have influenced vaccine efficacy. Firstly, the strain of bacteria, the antigen concentration, the method of bacterial inactivation and the adjuvant used are all factors that influence the efficacy of bacterial vaccines, although there are limited data on how these affect *M. bovis* vaccines in particular. Although some of these data are not reported in the above studies, and some are not available for our vaccine (e.g. the adjuvant used is proprietary), it is likely that all these factors varied among our study and those listed above. Secondly, calves in the above studies were first vaccinated at a substantially older age than the calves in our study. Immune responses of the newborn calf have unique characteristics and undergo rapid changes during the first few weeks of life [33]. Vaccination at 3, 14 and 35 days of age (as was performed in our study) may not elicit the same type of immune response as vaccination at 3 weeks of age (as in the Nicholas et al. [26] study, above). Our vaccination protocol was chosen based on (a) protocols that were being applied on dairies in Florida, and (b) the early age of infection that had been observed in previous studies [1]. Thirdly, calves in endemically infected herds in our study became colonized at a very early age, meaning that infection was likely well established before a vaccine-induced immune response could develop. Adaptive immune responses that develop after infection are very inefficient at clearing mycoplasmal infections and often result in detrimental chronic inflammatory responses, and this could contribute to vaccine failure when animals are already infected at the time of vaccination. Lastly, the challenge load of *M. bovis* that calves are exposed to can affect the efficacy of vaccination. Given the high incidence of clinical mycoplasmal disease (60% of calves treated for *M. bovis*-associated disease) and the early age of colonization

observed in our endemically infected herds, the level of *M. bovis* challenge that calves were exposed to may have been significantly greater than that of the calves in other vaccine studies [23,26,27].

Vaccinated calves in one herd in our study had a greater risk of otitis media than did control calves. The risk of otitis media in control calves in Herd B seemed substantially less than that in Herd C, but examination of calf health records from previous years in Herd B showed that the risk of otitis media observed in control calves was similar to that which had been historically present (data not shown). Therefore, vaccination seemed to exacerbate clinical otitis media in this herd. There are other reports of exacerbation of clinical disease following *M. bovis* vaccination [30,31,38]. However, the immune mechanisms associated with adverse outcomes after *M. bovis* vaccination have not been determined.

Vaccination of calves did stimulate a systemic humoral immune response, with an increase in serum IgG_1 being detectable after the third vaccination. A tendency towards Th2-biased IgG_1-dominated humoral responses has also been reported after infection of calves with *M. bovis* [39,40]. As IgG_2 is a much more effective opsonin for phagocytosis of *M. bovis* than is IgG_1, an IgG_1 response may be relatively ineffective for control of *M. bovis* respiratory infections [41]. It is somewhat puzzling that a humoral response to infection was not obvious in control calves in Herd B where there was a high incidence of *M. bovis*-associated disease. Statistical comparison of IgG_1 responses in control groups in Herds A and B was not conducted. However, it appears that in the control group in Herd A, post-colostral IgG_1 antibody levels continued to decline throughout the study period (see Fig. 2), whereas in the control group in Herd B, they did not decline after 7 weeks of age. This result may reflect continued stimulation of the immune response as a result of the endemic nature of *M. bovis* in this herd. Additionally, *M. bovis* infection can result in local mucosal antibody responses without eliciting a substantial systemic humoral response [27]. Other investigators have also noted a poor correlation between serum antibody responses and *M. bovis* infection in individual calves during the first 3 months of life [42].

The vaccine used in our study was ineffective at preventing upper respiratory tract colonization with *M. bovis* in calves, even when colonization occurred after a humoral immune response to vaccination was established. With the exception of one calf, nasal colonization was not detected in calves in Herd A until 12 weeks of age, whereas a significant increase in serum IgG_1 responses to vaccination was evident by 7 weeks of age. This is consistent with other reports on *M. bovis* vaccines; even where *M. bovis* vaccines have been associated with clinical benefits, they typically fail to induce an immune response that prevents upper respiratory tract infection [26,29]. Our findings are consistent with the idea that protection from *M. bovis* infection is better correlated with local mucosal immune responses than with serum antibody titers [27].

Total protein concentrations or *M. bovis*-specific antibody levels in post-colostral serum were not associated with protection from *M. bovis*-associated disease in calves in this study. However, as colostrum was not pasteurized on these farms, it is possible that some colostrum containing high antibody concentrations to *M. bovis* may have come from cows with intramammary infection and therefore may also have contained live *M. bovis*. This could certainly mask any protective effect of passive transfer when assessed on a herd level. Further studies are required to determine the efficacy of passive transfer for prevention of *M. bovis*-associated disease in a controlled setting.

To the best of the authors' knowledge this is the first peer-reviewed, controlled, independent efficacy study of any of the *M. bovis* vaccines available in North America. Vaccination was not efficacious in preventing *M. bovis*-associated disease in pre-weaned calves in two endemically infected Florida dairy herds, nor was it effective at preventing colonization of the upper respiratory tract in older calves in a third herd. The vaccine did stimulate a systemic IgG_1 response that was detectable after the third vaccination, but most clinical disease occurred prior to this response. Calves in these endemically infected herds were colonized with *M. bovis* at a very young age, and it is likely that this represents the greatest impediment to successful vaccination in this age group. The vaccine used in this field trial was formulated for use in much older cattle (stocker and feeder calves) for the prevention of respiratory disease, and the results of this study should not be extrapolated to infer whether vaccination is efficacious in that age group. Our findings highlight the importance of targeting vaccines for use in young calves specifically to this age group, and illustrate some of the challenges to the development of an efficacious vaccine against *M. bovis* for use in very young calves, if vaccination of this age group is even possible.

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