Superior protection from live-attenuated vaccines directed against Johne’s disease.

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Running title: Johne’s disease vaccines in goats

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Abstract

*Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) is the etiological agent of Johne’s disease in ruminants. Johne’s disease is an important enteric infection causing large economic losses associated with infected herds. In an attempt to fight this infection, we created two novel live-attenuated vaccine candidates with mutations in *sigH* and *lipN* (*pgsH* and *pgsN*, respectively). Earlier reports in mice suggested these vaccines are promising candidates to fight Johne’s disease in ruminants. In this study, we tested the performance of the two constructs as vaccine candidates using the goat model of Johne’s disease. Both vaccines appeared to provide significant immunity to goats against challenge from wild-type *M. paratuberculosis*. The *pgsH* and *pgsN* constructs showed significant reduction in histopathological lesions and tissue colonization compared to non-vaccinated and those vaccinated with an inactivated vaccine. Unlike the inactivated vaccine, the *pgsN* construct was able to eliminate fecal shedding from challenged animals, a feature that is highly desirable to control Johne’s disease in infected herds. Furthermore, strong, initial cell-mediated immune responses were elicited in goats vaccinated with *pgsN* that were not demonstrated in other vaccine groups. Overall, results indicate the potential use of live-attenuated vaccines to control intracellular pathogens, including *M. paratuberculosis*, and warrant further testing in cattle, the main target for Johne’s disease control programs.

**Keywords:** Johne’s disease, protective immunity, live-attenuated vaccine, adjuvant
Introduction

Johne’s disease (JD) is a gastrointestinal infection of ruminants caused by Mycobacterium avium subspecies paratuberculosis (M. paratuberculosis) (1). In the United States, JD is responsible for significant economic impact in the dairy cattle industry with estimated annual losses of $200 to $500 million mainly due to reduced milk production and low, but persistent mortality (2). The prevalence of JD in United States dairy operations is estimated to be greater than 90% (3). JD is also a public health concern as M. paratuberculosis infections have been associated with Crohn’s disease patients (4, 5). These studies highlight the need to establish JD control programs to benefit the livestock industry and human health. The most logical JD control measure in ruminants is vaccination, but development of a quality vaccine has proven difficult. To date, only three JD vaccines are commercially available, Silirum® (Pfizer), Gudair® (CZ Veterinaria), and Mycopar® (Boehringer Ingelheim), all of which are inactivated whole cell vaccines. These vaccines induce a severe injection site inflammatory reaction and also interfere with diagnostic tests to identify Mycobacterium bovis infected animals (6-8). More importantly, they do not provide adequate protection, and do not prevent M. paratuberculosis fecal shedding (9, 10). These drawbacks emphasize the need to develop a new class of vaccines that can efficiently control JD.

The most important component of an ideal JD vaccine is to provide long-term protection against M. paratuberculosis. Live-attenuated vaccines (LAV) are considered advantageous because they are easy to produce and manufacture, and have been shown to stimulate cell-mediated immunity in mice and ruminant models of JD (11-15). To develop our novel LAV candidates, our group employed a whole-genome expression profile of M. paratuberculosis exposed to different environmental stress conditions, or shed in cow feces, to identify new
virulence genes (16). These stress studies identified a sigma factor, \( \text{sigH} \), that was differentially co-regulated with a large number of genes based on the type of stressor used, and a fatty acid degradation lipase/esterase, \( \text{lipN} \), which was significantly up-regulated in \textit{M. paratuberculosis} shed in cow feces (16). Further analysis showed a \( \text{lipN} \) deletion mutant (hereby referred to as pgsN) was attenuated in mice as shown by reduced histopathological lesions and colonization of the liver (16). Subsequent studies with a \( \text{sigH} \) deletion mutant (hereby referred to as pgsH) showed attenuation in mice and induction of superior protective immune responses both with and without the saponin-based QuilA adjuvant (15, 17). In the present study, we compared key parameters desired for a successful JD vaccine (robust immune responses, reduced fecal shedding and reduced tissue damage) when novel LAV formulations were used in comparison to a commercially available vaccine, Mycopar\textsuperscript{®}, or no vaccine, in a goat challenge model of JD.

**Materials and methods**

**Animals**

A total of 24 male baby goats (kids), approximately 3 weeks old, were obtained from a local farm with no previous history of JD where all pregnant dams tested negative for JD by MAP serum ELISA (Paracheck\textsuperscript{®}, Biocor Animal Health, Omaha, NE). The kids were moved to animal facilities at the University of Wisconsin-Madison, acclimated for 1 week, and randomly assigned to one of the study groups. MAP serum ELISA (Paracheck\textsuperscript{®}) was performed on all kids to ensure their JD-free status before inclusion in the study. Furthermore, environmental fecal samples were randomly collected from each pen and were found to be negative for MAP by culture and IS\textit{900} PCR. Kids assigned to the naïve control group were housed in a different location than the other groups, and no contact was allowed between the naïve and challenged
5 groups at any time during the study. All animal care and experimental procedures were conducted in compliance to the protocols approved by the Institutional Animal Care and Use Committee, University of Wisconsin-Madison.

**Study design**

Goats were randomly separated into 5 groups. The experimental groups were vaccinated with sterile phosphate-buffered saline (PBS) (n=4), Mycopar® (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) (n=4), pgsH (n=6), or pgsN (n=6). A naïve control group (n=4) was housed in a separate location than the other groups. All goats in the experimental groups were challenged 60 days after vaccination. Blood and fecal samples were collected monthly throughout the study for immunological and bacteriology analyses, as detailed below.

Intradermal skin tests were performed on all goats at 60 days post-vaccination (DPV), 6 months post-challenge (MPC), and 12 MPC. All goats were euthanized and necropsied at 12 MPC for bacteriology and histopathology analyses (Fig. 1).

**Vaccination and challenge of goats**

The inactivated Mycopar® vaccine was administered as a single dose according to manufacturer’s instructions by subcutaneous injection. The live-attenuated vaccine candidates, pgsH and pgsN vaccine constructs were created by homologous recombination as previously described (16, 17). The experimental vaccines were grown in 7H9 liquid media supplemented with 0.5% glycerol, 2 μg/ml mycobactin J (Allied Monitor, Fayette, MO), and 10% ADC (2% glucose, 5 % bovine serum albumin fraction V, and 0.85% NaCl) (18). All bacterial cultures were freshly cultivated for 8-12 weeks, and never refrigerated or frozen prior to use. Bacteria were pelleted by centrifugation at 3200 x g for 15 minutes at room temperature in a pre-weighed
50 ml conical tube. After washing, the inoculum was passed through a 27 gauge needle to break up clumped bacilli, the excess fluid was drained, and an accurate wet weight of the bacterial pellet was determined where 100 mg pelleted wet weight equals approximately 1x10^9 CFU (19).

The inoculum concentration was confirmed by reading the optical density (OD) 600 nm and by dilution plating on 7H10 Middlebrook agar supplemented with 0.5% glycerol, 2 μg/ml mycobactin J (Allied Monitor), and 10% ADC. A 1 mg/ml stock solution of QuilA® adjuvant (Desert King, San Diego, CA) was prepared in PBS and filter sterilized. After the goats had acclimated for 7 days, each goat received a single dose of 1x10^9 CFU suspended in 1 ml sterile PBS containing 100 μg QuilA adjuvant. The experimental vaccines were administered by subcutaneous injection in the lower right side of the neck. At 60 days post vaccination, all goats (except naïve control) were challenged by oral infection with clinical strain M. paratuberculosis JTC 1285 (20). Bacteria were grown, processed, and quantified as described above for the vaccine strains. Each kid was allowed to nurse the inoculum (1x10^9 CFU in 10 ml milk replacer) from a syringe. Similar doses of the inoculum were prepared and given throughout the week for a total of 3 doses (total of approximately 3x10^9 CFU).

**Fecal and tissue culture**

Environmental and individual fecal samples collected from goats were processed using a previously described sedimentation method with minor variations in an attempt to optimize *M. paratuberculosis* recovery (18). Briefly, 3 g of each fecal sample were weighed and homogenized with 30 ml 0.75% hexadecyl pyridinium chloride (HPC) (Sigma Aldrich, St. Louis, MO) overnight to reduce non-mycobacterial contaminants. The next day, supernatants were harvested and centrifuged at 3000 x g for 15 minutes to obtain mycobacterial pellets (designated as the 1 x g sample). Next, the settled feces was centrifuged at 1000 x g for 15 minutes with the
supernatants harvested and centrifuged at 3000 x g for 15 minutes to obtain mycobacterial pellets (designated as the 1000 x g sample). All of the pellets were washed twice and resuspended in PBS, with the 1 x g sample resuspended in 1 ml and the 1000 x g sample resuspended in 0.5 ml.

Tissue specimens of tonsil, liver, spleen, jejunum, duodenum, ileum, mesenteric lymph node, hepatic lymph node, prescapular lymph node, ileocecal lymph node, and ileocecal valve were obtained at necropsy for mycobacterial culture. Each specimen was trimmed and weighed to 2 grams, placed in whirl pack bags with 10 ml PBS, and homogenized in a Seward Stomacher for 10 minutes on high. For plating, a 0.1 ml aliquot of the fecal supernatants and tissue homogenates (as well as a 1:10 dilution of the homogenates) were plated on Middlebrook 7H10 agar supplemented with 0.5% glycerol, 2 µg/ml mycobactin J (Allied Monitor), and 10% ADC (18). Additional antibiotic supplementation with amphotericin B (50 µg/ml), vancomycin (100 µg/ml), and nalidixic acid (100 µg/ml) was used to prevent non-mycobacterial contamination. All plates were incubated at 37°C for 3 months.

**Intradermal skin test**

The intradermal skin test was performed on all goats at 60 DPV, 6 MPC, and 12 MPC. Standard *M. avium*, *M. bovis*, and Johnin PPD were obtained from the National Veterinary Services Laboratory (Ames, IA). A 0.1 ml aliquot of each PPD was administered by intradermal injection using standard tuberculin syringes on freshly clipped skin of the cervical neck region. Injection sites were pre-measured and marked with a black marker for easier determination of injection site location. The response to the PPD injections (skin thickness/induration) was measured using digital calipers at 72 hours post-injection.

**Gross pathology and microscopic lesions**
A complete detailed necropsy was performed on each animal by a board-certified veterinarian and veterinary pathologist. Tissue specimens obtained at necropsy were fixed in 10% buffered formalin, processed routinely, embedded in paraffin blocks, and stained with H&E or acid-fast stains (21). A lesion rating system was applied to the gross and histologic descriptions for each animal including individual lesion severity scores for gross and microscopic lesions, and relative number of acid-fast bacilli (Table S1) (19).

**Antibody response**

Whole blood (5 ml) was collected from the jugular vein of each goat, placed in serum blood collection tubes (Becton, Dickinson and Co., Franklin Lakes, NJ), and centrifuged at 3000 x g for 10 minutes for serum collection. Serum collected from goats was tested for antibodies to *M. paratuberculosis* by ELISA. ELISA testing was performed according to manufacturer’s instructions of a USDA-approved, commercially available *M. paratuberculosis* ELISA testing kit for goats (Paracheck®, Biocor Animal Health, Omaha, NE). In cases of antibody saturation, sera were diluted and multiplied by the dilution factor to obtain an OD reading.

**Interferon-γ (IFN-γ) release assay**

Peripheral blood mononuclear cells (PBMCs) were isolated from goats as described previously (22). Briefly, 5 ml whole blood was collected from the jugular vein of each goat into EDTA Vacutainer tubes (Becton, Dickinson and Co.). A total of 3.5 ml whole blood was added to 3.5 ml HYCLONE RPMI-1640 (GE Life Sciences, Logan, UT) and mixed by inversion. This mixture was carefully layered onto 7 ml of Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO) in a 15 ml tube. After centrifugation at 400 x g for 30 minutes at room temperature, the layer containing PBMCs was removed to a new tube and washed twice with RPMI-1640 by...
centrifugation at 300 x g for 10 minutes at 4°C. The pellet was resuspended in 1 ml 0.83% ammonium chloride and incubated for 2-4 minutes to lyse any erythrocytes. RPMI-1640 was added for a final wash and the resulting pellet was resuspended in complete medium (RPMI-1640 with 2 mM L-Glutamine, 10% heat-inactivated fetal bovine serum (HIFBS), 1X non-essential amino acids (Gibco MEM NEAA 100X, Thermo Fisher Scientific, Waltham, MA), penicillin (100 IU/ml) and streptomycin (100 µg/ml)).

A total of 1x10⁶ PBMCs per well were stimulated with 10 µg/ml Johnin PPD for 72 hours. IFN-γ levels were measured in culture supernatants using a monoclonal antibody-based sandwich ELISA (Bovigam®, Biocor Animal Health, Omaha, NE), as per manufacturer’s instructions. The plate was developed in the dark until the OD 650 nm of the positive control was approximately 0.35. Following addition of the stop solution, the plate was read at OD 450 nm. The OD results are expressed as ELISA index values by dividing the mean OD of the PPD-stimulated supernatant by the mean OD of the corresponding unstimulated supernatant. When needed, supernatants were diluted, and multiplied by the dilution factor before an ELISA index value was calculated with the formula described above.

**Statistical analysis**

Differences between the groups were analyzed using a Student’s t-test. A probability value of less than 0.05 was considered significant for all tests. All statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

**Results**

**Safety of live-attenuated vaccine candidates**
In order to characterize the safety of LAV candidates, we regularly monitored vaccine shedding, body weight, body temperature, body condition, and injection site lesions in all animals. One of the goats in the Mycopar® group died one month after immunization due to sickness unrelated to JD. Minimal shedding of the vaccine strains was seen in the feces and saliva of the LAV candidates as determined by PCR (Table S2) and culture (Table S3). As expected, no change in body temperature, body condition or body weight was observed amongst the groups (data not shown). However, injection site lesion measurements, showed large skin indurations in all goats vaccinated with Mycopar®, most likely caused by the mineral oil adjuvant. Interestingly, Mycopar® associated skin lesions were most evident after challenge and lasted throughout the duration of the study. In contrast, pgsN vaccinated goats showed an initial, strong reaction at the injection site which quickly subsided, and minimal skin indurations were observed in pgsH vaccinated goats shortly after vaccination, suggesting an advantage to the attenuated vaccine formulations (Fig. 2).

Immune responses elicited by LAV candidates

In order to evaluate the immune responses elicited by LAV candidates, we analyzed key markers of cell-mediated and humoral immunity used for JD vaccine testing. Analysis of cell-mediated immunity as shown by IFN-γ levels with the commercial Bovigam® ELISA kit displayed a significant IFN-γ response in pgsN ($p<0.01$) and Mycopar® ($p<0.01$) vaccinated goats when compared to the PBS group at 60 DPV. Although both significant, goats vaccinated with pgsN had an earlier, and higher, release of IFN-γ than the commercially available Mycopar® vaccine. Meanwhile, minimal IFN-γ levels were seen in goats vaccinated with the pgsH LAV candidate (Fig. 3). The early, robust induction of IFN-γ in pgsN vaccinated goats is a

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desired feature for a JD vaccine that could be given to ruminants in the first few weeks of life to
protect against *M. paratuberculosis* infection (23).

Additionally, we used the intradermal skin test as another assay to measure the induction
of overall cell-mediated immunity in goats. Comparative intradermal skin tests were performed
with *M. bovis* and Johnin PPD at 60 DPV (Fig. 4A), and *M. avium, M. bovis*, and Johnin PPD at
6 MPC (Fig. 4B) and 12 MPC (Fig. 4C). Strong responses to *M. avium* PPD were seen in goats
vaccinated with Mycopar® and pgsN with minimal responses seen in the PBS, naïve, and pgsH
groups. Most importantly, the pgsN vaccinated goats showed a significant (*p*<0.001) response to
Johnin PPD injection compared to the PBS group prior to challenge (60 DPV). In comparison,
Mycopar® vaccinated goats also showed a significant (*p*<0.001) response to Johnin PPD
compared to the PBS group. The pgsH LAV candidate did not show any significant response to
Johnin PPD when compared to the PBS group at any time-point. *M. bovis* PPD testing, however,
did show a response in pgsN vaccinated goats at 60 DPV, but was not shown at 6 or 12 MPC. *M.
bovis* testing is an important feature of JD vaccines in cattle, and further testing is needing to
address whether *M. bovis* infected animals can respond to PPD of *M. paratuberculosis* or the
pgsN LAV candidate.

Finally, we measured the JD antibody response using the commercial Paracheck® ELISA
kit to evaluate *M. paratuberculosis* specific IgG. As expected, the Mycopar® vaccinated goats
elicited a robust antibody response throughout the majority of the study, most likely due to the
paraffin oil adjuvant used in the vaccine formulation. No significant antibody response was seen
in either LAV candidate. Increased antibody levels in response to the *M. paratuberculosis* oral
challenge were seen in the PBS group starting at 7 MPC (Fig. 5).
M. paratuberculosis shedding from vaccinated animals

To gauge the ability of vaccine candidates to prevent spread of infection, we evaluated challenge strain fecal shedding, tissue colonization, and tissue histopathology in all goat groups. For fecal shedding, environmental fecal samples were collected for the first 8 MPC with individual fecal samples collected monthly thereafter. A terminal fecal sample was collected for each goat, and designated as the 13 MPC sample. Evaluation of M. paratuberculosis challenge strain fecal shedding in goats was determined by culture on solid media (Table 1). No M. paratuberculosis colonies were recovered from goat feces in the naïve group at any time during the study, further confirming their JD-free status. Meanwhile, goats in the PBS vaccinated group started to shed the M. paratuberculosis challenge strain in their feces at 7 MPC. Minimal to mild fecal shedding was seen in the pgsH and Mycopar® groups throughout the study. The pgsN vaccinated goats, however, did not have any detectable M. paratuberculosis challenge strain shedding, a key attribute of an effective JD vaccine.

M. paratuberculosis colonization following immunization

Goat tissues collected at necropsy were cultured on solid media to estimate the level of M. paratuberculosis challenge strain colonization in all animal groups. An emphasis was placed on intestinal tissue sections and associated lymph nodes due to the known pathogenic mechanisms of JD. As expected, no M. paratuberculosis colonies were detected in the tissues harvested from goats in the naïve control group. Few or no colonies were isolated from the tonsil, liver, spleen, hepatic lymph node, and prescapular lymph node for any of the groups that received oral infection of the challenge strain (data not shown). As expected, the PBS vaccinated group showed high levels of tissue colonization in the jejunum, duodenum, ileum, mesenteric
lymph node, ileocecal lymph node, and ileocecal valve (Fig. 6A-F). For pgsH, only the ileum (Fig. 6C), mesenteric lymph node (Fig. 6D), and ileocecal lymph node (Fig. 6E) showed significantly reduced bacterial colonization levels when compared to the PBS group. In contrast, goats vaccinated with Mycopar® did not show reduced bacterial colonization of any of the intestinal tissue, but did show significantly reduced bacterial colonization of the mesenteric lymph node (Fig. 6D) and ileocecal lymph node (Fig. 6E) when compared to the PBS group.

Most importantly, bacterial colonization levels of pgsN vaccinated goat tissues were significantly reduced in all of the intestinal tissues and associated lymph nodes assayed when compared to the PBS group (Fig. 6A-F). Furthermore, pgsN goat tissues were significantly reduced in the mesenteric lymph node (Fig. 6D) and ileocecal lymph node (Fig. 6E) when compared to the Mycopar® group.

M. paratuberculosis lesions in vaccine groups

Tissues taken at necropsy were also subjected to gross and microscopic pathology analysis by a board certified veterinary pathologist on a blind basis. As expected, no gross pathology or histopathological lesions associated with JD were seen in any of the naïve goat tissue. Gross pathology findings indicated moderate thickening of the intestinal mucosa, an indication of lymphocyte recruitment, in all of the challenged groups. The most obvious mucosal thickening was observed in the PBS group, with the least amount of thickening observed in the pgsN group. Prescapular lymph node enlargement and abscess formation were observed in Mycopar® vaccinated goats, with no such finding in any of the other groups. No characteristic signs of JD were observed in any of the groups upon gross examination of the liver, spleen, tonsil, and other lymph nodes. For the microscopic histopathological analysis, all goats from the PBS group displayed several small microgranulomas in the liver, with no multinucleate...
macrophages or larger granulomas observed. However, the jejunum had scattered small, patchy
aggregates of macrophages within the villi and lamina propria, with mild blunting of the villi
observed. Each jejunum specimen contained 2-3 microgranulomas without multinucleate
macrophages or larger granulomas. Acid-fast bacilli were rare, with only 1-2 detected per
jejenum specimen (Fig. 7A). The mesenteric lymph node specimens had marked medullary
histiocytosis (histiocytes=macrophages). All lymph node specimens had 50-60 microgranulomas
and granulomas with patchy foci of granulomatous inflammation that sometimes tended to
coalesce. Varying numbers of Langhans and foreign body multinucleate macrophages were
noted. Overall, samples collected from the PBS group exhibited moderate histopathological
lesions consistent with JD.

For Mycopar® vaccinated goats, liver specimens showed mild, multifocal Kupffer cell
hyperplasia with several small microgranulomas without associated multinucleate macrophages
or larger granulomas detected. Interestingly, jejunum specimens had occasional variable-sized
granulomatous foci, sometimes containing multinucleate macrophages within the lamina propria,
and scattered villi were blunted. Granulomatous foci in jejunum specimens contained variable
numbers of acid-fast bacilli (Fig. 7B). The mesenteric lymph nodes had mild medullary edema,
and moderate to marked medullary histiocytosis, with numerous scattered patchy aggregates of
macrophages near the corticomedullary junction. Other lymph nodes examined showed similar
changes. Overall, animals vaccinated with Mycopar® showed lesions consistent with JD
infection, but to a lesser extent than animals in the PBS group.

For pgsH vaccinated goats, liver sections showed minimal to mild Kupffer cell
hyperplasia with very few microgranulomas, and acid fast bacilli were detected. No
multinucleate macrophages or larger granulomas were observed. Most jejunum specimens had
moderate dilation of submucosal lymphatics. No microgranulomas, granulomas, or acid-fast bacilli were observed in any of the jejunum specimens (Fig. 7C). The mesenteric lymph nodes had moderate medullary histiocytosis with scattered, patchy aggregates of macrophages near the corticomedullary junction. Very few microgranulomas were present within the cortex of each specimen. No multinucleate macrophages, large granulomas, or acid-fast bacilli were detected. Similar lesions were observed in the other lymph nodes, excluding the prescapular lymph node. Overall, mild lesions associated with JD were detected in goats vaccinated with pgsH. For pgsN vaccinated goats, liver sections contained mild, multi-focal Kupffer cell hyperplasia with a very limited number of small microgranulomas detected. No multinucleate macrophages or large granulomas were observed. Jejunum specimens had mildly dilated submucosal lymphatics and occasional dilated villous lacteals, but no microgranulomas, large granulomas, or acid-fast bacilli were detected (Fig. 7D). The mesenteric lymph node specimens had marked, diffuse medullary histiocytosis with numerous scattered, patchy aggregates of macrophages or small granulomas detected within the cortex. Scattered lymphoid follicles had moderate lymphoid depletion, but no well-defined microgranulomas, granulomas, or clear acid-fast bacilli were detected. Overall, mild lesions associated with JD infection were observed, with similar lesion scores to pgsH. In general, pgsH and pgsN vaccinated groups displayed fewer lesions associated with M. paratuberculosis compared to Mycopar® or un-vaccinated groups (Fig. S1).

Discussion

Vaccination is considered the most logical control strategy for controlling JD in ruminants. Implementing an effective JD vaccination regimen, however, has proven difficult despite the Mycopar® commercial vaccine available for cattle, and the Gudair® and Silirum® vaccines commercially available for smaller ruminants. Previous studies with these inactivated
vaccines suggest protective efficacy as shown by reduced fecal shedding, increased average milk production, and delayed signs of clinical disease (24-27). Despite these positive attributes, these inactivated vaccines have shown limited ability to prevent new JD infection within a ruminant herd (28). Due to this limitation, alternative forms of vaccine development have been pursued in an effort to increase the use of JD vaccines in control programs. Recent studies suggest LAVs as a strategy for creating more effective JD vaccine candidates (11-15, 19). The primary goal of this study was to examine the performance of live-attenuated vaccines (represented by pgsH and pgsN) as a viable alternative to inactivated vaccines (represented by Mycopar®) to control Johne’s disease in dairy herds. For this purpose, we used the caprine model that was used previously (12, 19, 22). Unfortunately, it was difficult to identify a goat herd that was Johne’s disease free for our study which resulted in reducing the group size for the Mycopar® vaccine, a well-studied vaccine (12, 29, 30). However, we were able to successfully identify significant differences between experimental groups, despite this limitation. In addition, a group that received only PBS (control group), but challenged 2 months later, displayed the expected intermittent fecal shedding, another confirmation of the validity of the used challenge protocol.

The main problems associated with the currently licensed JD vaccines include the development of skin lesions at the site of vaccination, interference with *M. bovis* infection surveillance in ruminant herds and inability to reduce *M. paratuberculosis* shedding from infected animals (6-8). In this study, goats vaccinated with Mycopar® displayed large injection site skin lesions that lasted throughout the course of the study. In contrast, both our LAV candidates displayed mild to minimal injection site skin lesions for only a brief time following vaccination, a strong indication of the safety of both LAV candidates. One of our LAV candidates, pgsN, however, showed a response to *M. bovis* PPD skin testing at 60 DPV, but not
at 6 or 12 MPC. Additionally, skin tests with *M. avium* PPD showed a response in pgsN and Mycopar® vaccinated goats at various times throughout the study. Results like these are not uncommon, since high exposure to environmental mycobacteria can occur throughout the United States. This exposure could provide a plausible explanation for varying skin test and IFN-γ test results (19). Further investigation is needed to determine whether our LAV candidates will interfere with *M. bovis* skin testing. Moreover, the development of an assay to differentiate infected from vaccinated animals (DIVA) could further help the deployment of a pgsN vaccine in the field.

Both PGS LAV candidates, and Mycopar®, elicited immune responses that were able to limit challenge strain tissue colonization, fecal shedding, and histopathological lesions in goats when compared to the PBS vaccinated group. The pgsN LAV, however, displayed a more significant reduction in challenge strain tissue colonization and completely eliminated fecal shedding, compared to the other two previously mentioned vaccines. Histopathological analysis further confirmed the superiority of protection of pgsN by reducing the number of microgranulomas, while both pgsH and Mycopar® groups displayed higher number of microgranulomas, respectively. Although both Mycopar® and pgsN elicited strong IFN-γ responses in goats, the immediate, robust IFN-γ response seen in pgsN vaccinated goats is a plausible explanation for the significant protective efficacy observed in our study. These findings are validated based on previous studies, where reduced levels of IFN-γ correlated to the development of clinical disease (23, 31). Furthermore, IFN-γ has been identified as an important cytokine in controlling numerous types of infections caused by mycobacteria (32).

In summary, both LAV candidates provided some protection to goats against virulent *M. paratuberculosis* challenge. However, the pgsN LAV showed significant protective efficacy as
shown by the elimination of challenge strain fecal shedding, reduced histopathological lesions, and significantly reduced challenge strain tissue colonization. The immediate, robust IFN-γ response seen in pgsN vaccinated goats is hypothesized to be a major contributor to the significant protective efficacy observed in this study. In contrast, immune responses in goats generated by Mycopar® vaccine were only partially effective in limiting tissue colonization and fecal shedding. Overall, our data suggest the pgsN LAV candidate warrants a more systemic analysis in the calf model of JD.

**Funding information**

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References


with recombinant IL-12 in a bovine experimental infection model. Vaccine 21:3101-3109.


Table 1. *M. paratuberculosis* challenge strain fecal shedding (CFU/g) means and ranges\(^a\)

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<th>Months Post-Challenge</th>
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<tr>
<td>pgsH</td>
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<tr>
<td>Mycopar(^b)</td>
<td>7 (0-13)</td>
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<td>pgsN</td>
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\(^a\) A 3 CFU limit of detection was used. No *M. paratuberculosis* colonies were recovered from any of the goats in the naïve group at any time-point and hence were not represented here.
**Figure legends**

**Fig. 1.** Timeline for experimental vaccination and challenge used in this study. Kids, 28 days old, were vaccinated by subcutaneous injection and challenged 60 days later with a virulent strain of *M. paratuberculosis* JTC 1285 by oral inoculation of bacteria in milk.

**Fig. 2.** Skin lesions following vaccination. Vaccine injection site lesions were measured throughout the duration of the study. Data are expressed as arithmetic means with the error bars representing the standard experimental mean. *p* < 0.05 indicates significant skin lesions comparing Mycopar® to pgsN and pgsH. -1 represents the 30 DPV time-point and 0 is the 60 DPV time-point just prior to challenge. Data points prior to -1 indicate data collected between 1 and 30 DPV.

**Fig. 3.** IFN-γ response in goat groups before and after vaccination. PBMCs were isolated from whole goat blood and stimulated with Johnin PPD for 72 hours. IFN-γ levels in culture supernatants were determined using a Bovigam® ELISA kit. Data are expressed as an ELISA index with error bars representing the standard experimental mean with *p* < 0.05 when compared to the PBS group. -1 represents the 30 DPV time-point and 0 is the 60 DPV time-point just prior to challenge.

**Fig. 4.** Intradermal skin test performed on goats at 60 DPV (**A**), 6 MPC (**B**), and 12 MPC (**C**). Each graph shows the pre-injection skin thickness measurement (0 hour) and the skin thickness measurement 72 hours after PPD injection. Each data point represents an individual animal while the horizontal lines indicate the mean value for each group with *p* < 0.05 when compared to the PBS group. The 0 hour skin thickness was subtracted from the 72 hour skin thickness prior to statistical analysis.

**Fig. 5.** Antibody response in goat groups before and after vaccination using ELISA. -2 is baseline pre-vaccination bleeding. -1 and 0 represent samples collected at 30 DPV and 60 DPV, respectively. Error bars represent the standard experimental mean with *p* < 0.05 when compared to the PBS group.
Fig. 6. *M. paratuberculosis* challenge strain colonization of goat tissues. Graphs represent tissue samples taken from jejunum (A), duodenum (B), ileum (C), mesenteric LN (D), ileocelecal LN (E), and ileocelecal valve (F). Error bars indicate standard deviation with *p*<0.05 and **p**<0.01. There were no *M. paratuberculosis* colonies recovered from any tissues harvested from goats in the naïve group.

Fig. 7. Histopathology of liver (left) and jejunum (right) samples from goat groups following challenge with *M. paratuberculosis* JTC 1285. Tissues stained with hematoxylin and eosin collected from PBS (A), Mycopar® (B), pgsH (C), and pgsN (D) vaccinated goats 12 MPC. Arrows indicate granuloma infiltrates. Ziehl-Neelson stained jejunum samples are also included with arrow heads indicating acid-fast bacilli. No JD-associated granuloma infiltrates or acid-fast bacilli were found in any tissues in the naïve group.
Figures

Fig. 1.

Goats

Vaccinate

Challenge

Sacrifice & Collect Tissues

28 days old

60 days

Collect blood and fecal samples monthly

Months

0 1 2 3 4 5 6 7 8 9 10 11 12
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.