Superior Protection from Live-Attenuated Vaccines Directed against Johne’s Disease

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ABSTRACT Mycobacterium avium subsp. 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 performances 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 a significant reduction in histopathological lesions and tissue colonization compared to nonvaccinated goats 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, the 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, pathogenesis

Johne’s disease (JD) is a gastrointestinal infection of ruminants caused by Mycobacterium avium subsp. 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 rates (2). The prevalence of JD in U.S. 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 the 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 they 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, sigH, that was differentially coregulated with a large number of genes based on the type of stressor used, and a fatty acid degradation lipase/esterase, lipN, which was significantly upregulated in M. paratuberculosis shed in cow feces (16). Further analysis showed that a lipN deletion mutant (here referred to as pgsN) was attenuated in mice, as shown by reduced histopathological lesions and colonization of the liver (16). Subsequent studies with a sigH deletion mutant (here 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, or no vaccine, in a goat challenge model of JD.

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 (Fig. 1). One of the goats in the Mycopar group died 1 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 among 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 interferon gamma (IFN-γ) levels with the commercial Bovigam enzyme-linked immunosorbent assay (ELISA) kit, displayed a significant IFN-γ response in pgsN-
vaccinated ($P < 0.01$) and Mycopar-vaccinated ($P < 0.01$) goats compared to the phosphate-buffered saline (PBS) group at 60 days postvaccination (DPV). Although both results were statistically significant, goats vaccinated with pgsN had an earlier and greater release of IFN-$\gamma$ than goats vaccinated with the commercially available Mycopar vaccine. Meanwhile, minimal IFN-$\gamma$ levels were seen in goats vaccinated with the pgsH LAV candidate (Fig. 3). The early robust induction of IFN-$\gamma$ in pgsN-vaccinated goats is a 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 (18).

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 purified protein derivative (PPD) at 60 DPV (Fig. 4A) and *M. avium, M. bovis,* and Johnin PPD at 6 months postchallenge (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, naive, 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 compared to the PBS group at any time point. *M. bovis* PPD testing, however, did show a response in pgsN-vaccinated goats. 

![Skin lesions following vaccination](image1)

**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.

![IFN-$\gamma$ response in goat groups before and after vaccination](image2)

**FIG 3** IFN-$\gamma$ response in goat groups before and after vaccination. PBMCs were isolated from whole goat blood and stimulated with Johnin PPD for 72 h. IFN-$\gamma$ 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; *, $P < 0.05$ compared to the PBS group. −1 represents the 30 DPV time point, and 0 is the 60 DPV time point just prior to challenge.
goats at 60 DPV but not 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).

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

**FIG 5** Antibody response in goat groups before and after vaccination using ELISA. −2 is baseline prevaccination bleeding, and −1 and 0 represent samples collected at 30 DPV and 60 DPV, respectively. Error bars represent the standard experimental mean; *, *P* < 0.05 compared to the PBS group.
TABLE 1  *M. paratuberculosis* challenge strain fecal shedding

| Vaccination group | Fecal shedding (CFU/g) by month postchallenge (mean [range]) |
|-------------------|-------------------------------------------------------------|
|                   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  |
| PBS               | 0 (0–0) | 0 (0–0) | 5 (0–10) | 7 (0–13) | 10 (0–20) | 0 (0–0) | 22 (13–30) | 55 (33–127) | 63 (33–127) | 116 (0–463) | 6 (0–13) | 22 (0–47) | 15 (0–60) |
| pgsH              | 0 (0–0) | 0 (0–0) | 20 (0–40) | 9 (0–17) | 40 (0–80) | 0 (0–0) | 5 (0–10) | 5 (0–10) | 17 (0–40) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 6 (0–37) |
| Mycopar           | 7 (0–13) | 9 (0–17) | 0 (0–0) | 5 (0–10) | 5 (0–10) | 0 (0–0) | 19 (0–37) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) |
| pgsN              | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) |

*A 3 CFU limit of detection was used. No *M. paratuberculosis* colonies were recovered from any of the goats in the naive group at any time point; hence, they are not represented here.

*M. paratuberculosis* shedding from vaccinated animals. To gauge the ability of vaccine candidates to prevent the 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 the 13 MPC sample. Evaluation of *M. paratuberculosis* challenge strain fecal shedding in goats was determined by culture on solid medium (Table 1). No *M. paratuberculosis* colonies were recovered from goat feces in the naive 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 medium 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, or 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 to 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 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) 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 compared to the PBS group (Fig. 6A to F). Furthermore, pgsN goat tissues were significantly reduced in the mesenteric lymph node (Fig. 6D) and ileocecal lymph node (Fig. 6E) 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 tissues. 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 micro-
scopic 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 to 3 microgranulomas without multinucleate macrophages or larger granulomas. Acid-fast bacilli were rare, with only 1 to 2 detected per jejunum specimen (Fig. 7A). The mesenteric lymph node specimens had marked medullary histiocytosis (histiocytes = macrophages). All lymph node specimens had 50 to 60 microgranulomas and granulomas with patchy foci of granulomatous inflamma-

FIG 6 M. paratuberculosis challenge strain colonization of goat tissues. Graphs represent tissue samples taken from jejunum (A), duodenum (B), ileum (C), mesenteric lymph nodes (LN) (D), ileocecal LN (E), and ileocecal valve (F). Error bars indicate standard deviation; *, $P < 0.05$; **, $P < 0.01$. There were no M. paratuberculosis colonies recovered from any tissues harvested from goats in the naive group.
tion that sometimes tended to coalesce. Various 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 mac-

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-vaccinated (A), Mycopar-vaccinated (B), pgsH-vaccinated (C), and pgsN-vaccinated (D) goats 12 MPC. Arrows indicate granuloma infiltrates. Ziehl-Neelsen-stained jejunum samples are also included, with arrowheads indicating acid-fast bacilli. No JD-associated granuloma infiltrates or acid-fast bacilli were found in any tissues in the naive group.
rophages or larger granulomas detected. Interestingly, jejunal specimens had occasional granulomatous foci of various sizes, sometimes containing multinucleate macrophages within the lamina propria, and scattered villi were blunted. Granulomatous foci in jejunal specimens contained varied 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 multinucleated macrophages or larger granulomas were observed. Most jejunal specimens had moderate dilation of submucosal lymphatics. No microgranulomas, granulomas, or acid-fast bacilli were observed in any of the jejunal 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 multifocal Kupffer cell hyperplasia, with a very limited number of small microgranulomas detected. No multinucleate macrophages or large granulomas were observed. Jejunal 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 lesion scores similar to those of pgsH. In general, pgsH- and pgsN-vaccinated groups displayed fewer lesions associated with M. paratuberculosis compared to Mycopar or unvaccinated 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 that are 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 (19–22). Despite these positive attributes, these inactivated vaccines have shown limited ability to prevent new JD infection within a ruminant herd (23). 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, 24). 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, 24, 25). 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, 26, 27). 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 was 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 an 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 varied skin test and IFN-γ test results (24). 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 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 numbers 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 (18, 28). Furthermore, IFN-γ has been identified as an important cytokine in controlling numerous types of infections caused by mycobacteria (29).

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 that the pgsN LAV candidate warrants a more systemic analysis in the calf 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 history of JD and where all pregnant dams tested negative for JD by *M. paratuberculosis* serum ELISA (Paracheck; 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. *M. paratuberculosis* serum ELISA (Paracheck) 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 *M. paratuberculosis* by culture and IS900 PCR. Kids assigned to the naive control group were housed in a different location than the other groups, and no contact was allowed between the naive and challenged groups at any time during the study. All animal care and experimental procedures were conducted in compliance with 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 naive control group (n = 4) was housed in a separate location from 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 bacteriological analyses, as detailed below. Intradermal skin tests were performed on all goats at 60 days postvaccination (DPV), 6 months postchallenge (MPC), and 12 MPC. All goats were euthanized and subjected to necropsy at 12 MPC for bacteriological and histopathological analyses.

**Vaccination and challenge of goats.** The inactivated Mycopar vaccine was administered as a single dose, according to the manufacturer’s instructions, by subcutaneous injection. The live-attenuated vaccine candidates, the pgsH and pgsN vaccine constructs, were created by homologous recombination, as previously described (16, 17). The experimental vaccines were grown in 7H9 liquid medium 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) (30). All bacterial cultures were freshly cultivated for 8 to 12 weeks and never refrigerated or frozen prior to use. Bacteria were pelleted by centrifugation at 3,200 × g for 15 min at room temperature in a preweighed 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 of pelleted wet weight equals approximately 1 × 10⁸ CFU (24). The inoculum concentration was confirmed by reading the optical density at 600 nm (OD₆₀₀) 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 1 × 10⁸ CFU suspended in 1 ml of sterile PBS containing 100 μg of QuilA adjuvant. The experimental vaccines were administered by subcutaneous injection in the lower right side of the neck. At 60 days postvaccination, all goats (except naive control) were challenged by oral infection with clinical strain M. paratuberculosis JTC 1285 (31). Bacteria were grown, processed, and quantified as described above for the vaccine strains. Each kid was allowed to nurse the inoculum (1 × 10⁷ CFU in 10 ml of 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 3 × 10⁹ 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 (30). Briefly, 3 g of each fecal sample was weighed and homogenized with 30 ml of 0.75% hexadecyl pyridinium chloride (HPC) (Sigma-Aldrich, St. Louis, MO) overnight to reduce nonmycobacterial contaminants. The next day, supernatants were harvested and centrifuged at 3,000 × g for 15 min to obtain mycobacterial pellets (designated the 1 × g sample). Next, the settled feces were centrifuged at 1,000 × g for 15 min with the supernatants harvested and centrifuged at 3,000 × g for 15 min to obtain mycobacterial pellets (designated the 1,000 × g sample). All of the pellets were washed twice and resuspended in PBS, with the 1 × g sample resuspended in 1 ml and the 1,000 × 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 g, placed in Whirl-Pak bags with 10 ml of PBS, and homogenized in a Seward stomacher for 10 min 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) was plated on Middlebrook 7H10 agar supplemented with 0.5% glycerol, 2 μg/ml mycobactin J (Allied Monitor), and 10% ADC. A 1 mg/ml stock solution of vancomycin (100 μg/ml), and nalidixic acid (100 μg/ml) was used to prevent nonmycobacterial 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 premeasured and marked with a black marker for easier determination of the injection site location. The response to the PPD injections (skin thickness/induration) was measured using digital calipers at 72 h postinjection.

**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 hematoxylin and eosin (H&E) or acid-fast stains (32). 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) (24).

**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 3,000 × g for 10 min for serum collection. Serum collected from goats was tested for antibodies to M. paratuberculosis by ELISA. ELISA testing was performed according to the manufacturer’s instructions for a USDA-approved commercially available M. paratuberculosis ELISA 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.

**IFN-γ release assay.** Peripheral blood mononuclear cells (PBMCs) were isolated from goats, as described previously (25). Briefly, 5 ml of 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 of whole blood was added to 3.5 ml of 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 × g for 30 min at room temperature, the layer containing PBMCs was removed to a new tube and washed twice with RPMI 1640 by centrifugation at 300 × g for 10 min at 4°C. The pellet was resuspended in 1 ml of 0.83% ammonium chloride and incubated for 2 to 4 min to lysis 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], 1× nonessential amino acids [Gibco minimum essential medium (MEM) NEAA 100×; Thermo Fisher Scientific, Waltham, MA], penicillin [100 IU/ml], and streptomycin [100 μg/ml]).

A total of 1 × 10^5 PBMCs per well were stimulated with 10 μg/ml Johnin PPD for 72 h. IFN-γ levels were measured in culture supernatants using a monoclonal antibody-based sandwich ELISA (Bovigam; Biocor Animal Health, Omaha, NE), as per the manufacturer’s instruction. The plate was developed in the dark until the OD650 of the positive control was approximately 0.35. Following addition of the stop solution, the plate was read at OD450. 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 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).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/CVI.00478-16.

TEXT S1, PDF file, 0.05 MB.
TEXT S2, PDF file, 0.2 MB.

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