Title: Superior Protection Elicited by Live-Attenuated Vaccines in the Murine Model of Paratuberculosis.

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Running title: Protective role of live-attenuated *M. paratuberculosis* strains against Johne's disease.

Abstract.

Mycobacterium avium subspecies *paratuberculosis* (*M. paratuberculosis*) causes Johne's disease, a chronic enteric infection in ruminants with severe economic impact on the dairy industry in the USA and worldwide. Currently, available vaccines have limited protective efficacy against disease progression and does not prevent spread of the infection among animals. Because of their ability to elicit wide-spectrum immune responses, we adopted a live-attenuated vaccine approach based on a *sigH* knock-out strain of *M. paratuberculosis* ($\Delta sigH$). Earlier analysis of the $\Delta sigH$ mutant in mice indicated their inadequate ability to colonize host tissues, unlike the isogenic wild-type strain, validating the role of this sigma factor in *M. paratuberculosis* virulence. In the present study, we evaluated the performance of the $\Delta sigH$ mutant compared to inactivated vaccine constructs in a vaccine/challenge model of murine paratuberculosis. The presented analysis indicated that $\Delta sigH$ mutant with or without QuilA adjuvant is capable of eliciting strong immune responses (such as interferon gamma- γ , IFN- γ) suggesting their immunogenicity and ability to potentially initiate effective vaccine-induced immunity. Following a challenge with virulent strains of *M. paratuberculosis*, $\Delta sigH$ conferred protective immunity as indicated by the reduced bacterial burden accompanied with reduced lesions in main body organs (liver, spleen and intestine) usually infected with *M. paratuberculosis*. More importantly, our data indicated better ability of the $\Delta sigH$ vaccine to confer protection compared to the inactivated vaccine constructs even with the presence of oil-adjuvant. Overall, our approach provides a rational basis for using live-attenuated mutant strains to develop improved vaccines that elicit robust immunity against this chronic infection.

74 **Keywords:** *Mycobacterium avium* subsp. *paratuberculosis*; Johne's disease; sigma factor *sigH*;
75 live attenuated vaccine, adjuvant

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1. Introduction:

Mycobacterium avium subsp. *paratuberculosis* (*M. paratuberculosis*) is the causative agent of Johne's disease, also known as paratuberculosis, in animals. Johne's disease is a substantial problem in cattle and in particular to the dairy industry [1]. Johne's disease, a potentially fatal disease to the infected ruminants, is a chronic enteric infection and responsible for significant economic losses throughout the world [2-4]. To minimize the impact of paratuberculosis, control programs have been initiated to prevent the spread of disease. However, several factors caused progress towards understanding paratuberculosis to lag behind and have limited the success of controlling this widespread epizootic [5,6]. These factors include the difficulty associated with the slow growth rate of *M. paratuberculosis*, microbial resistance to harsh environments, lack of accurate diagnostic tools and ineffectiveness of the available vaccines. A recent report indicated that more than 90% of the dairy operations in the United States (USA) include cows that are infected with *M. paratuberculosis* [1] with severe economic losses estimated to be over \$500 million annually in the US dairy industry alone [7]. Currently, there are three commercially available whole cell inactivated vaccines used in Johne's disease control programs worldwide: Gudair (CZ Veterinaria) is mostly used in Europe and Australia [8]; Silirum (Zoetis) is used in New Zealand [9]; and Mycopar™ (Boehringer Ingelheim) is licensed to control Johne's disease in the USA.[10,11]. However, earlier vaccine efficacy trials evaluating whole-cell inactivated *M. paratuberculosis* vaccines including Mycopar™ suggested inability of the killed vaccines to completely block infection transmission cycle despite their capacity to reduce bacterial fecal shedding and disease pathogenesis [8,11-13].

One of the criteria of an ideal vaccine is to provide long-term protective immunity against paratuberculosis. Subunit vaccines based on single genes and proteins are able to elicit systemic

and mucosal immunity [14-16]. However, experimental infection trials using subunit vaccines did not show significant improvement in disease prevalence over other approaches as they failed to provide complete protection against *M. paratuberculosis* infection in calves [17,18]. Additionally, several booster vaccinations are required to achieve effective protective immunity [14,15]. On the other hand, live-attenuated vaccines (LAV) with a diverse antigenic repertoire were able to elicit protective mucosal and systemic immune responses against *M. paratuberculosis* by stimulating innate and adaptive immunity [19,20]. Moreover, manufacture of LAV is cost effective and easy to produce. An earlier study of *M. paratuberculosis* with a genetic deletion of *sigL* indicated the ability of this mutant to generate protective immunity in mice associated with the production of robust IFN- γ levels [19]. Others used a similar infection model in mice which showed a significant reduction in organs bacterial load following immunization with transposon-mutants of *M. paratuberculosis* [21,22]. In small ruminant infection model, two LAV candidates, $\Delta relA$ and $\Delta leuD$, were protective in goats and calves, and able to reduce bacterial fecal shedding [20,23,24], a hallmark for the development of Johne's disease in infected animals. An optimal live-attenuated vaccine for the successful control of paratuberculosis should reduce bacterial organ colonization and reduce fecal shedding of *M. paratuberculosis* and thereby, reduce the opportunity for disease transmission among susceptible animals.

To gain a better understanding of what makes *M. paratuberculosis* a successful pathogen, our group employed a whole-genome based "stressosome" profiling of this bacteria in response to different environmental stressors and identified a novel set of putative virulence determinants, including global gene regulators that may be important for *M. paratuberculosis* virulence [25]. We have shown that many global gene regulators such as sigma factors are activated following

macrophage infection and are part of a global virulence regulatory system providing resistance to the host bactericidal activities [19,26]. Both *M. paratuberculosis* $\Delta sigH$ and $\Delta sigL$ mutant strains had attenuated pathogenicity in mice, suggesting their role as important virulence factors. Moreover, when we evaluated a $\Delta sigL$ mutant strain as a live-attenuated vaccine using the murine model, it elicited protective immune responses against *M. paratuberculosis* infection [19] suggesting global gene regulator based live-attenuated vaccines could be a successful approach for developing an effective vaccine against Johne's disease. In an earlier report, a role for *sigH* as a mycobacterial global gene regulator was suggested in *M. paratuberculosis* [27]. In this study, we evaluated the protective efficacy of $\Delta sigH$ mutant as a live-vaccine candidate against challenge with the wild-type *M. paratuberculosis* K-10 strain as well as field isolates (*M. paratuberculosis* JTC1285). The generated immune responses and protective potential of $\Delta sigH$ mutant were evaluated in presence or absence of QuilA adjuvant and in comparison to inactivated vaccine constructs. Overall, the $\Delta sigH$ mutant elicited superior protective immune responses than other vaccine constructs, regardless the QuilA adjuvant is used for immunization or not.

2. Materials and methods

2.1. Bacterial strains and media.

Wild-type *Mycobacterium avium* subsp *paratuberculosis* (*M. paratuberculosis*) K10, clinical strain *M. paratuberculosis* JTC1285, and *M. paratuberculosis* $\Delta sigH$ mutant strains were cultured in Middlebrook media as previously described [3,19,26]. Briefly, *M. paratuberculosis* strains were grown at 37°C in Middlebrook 7H9 supplemented with 10% ADC (111 mM glucose, 5% BSA, 124 mM NaCl), 0.5% glycerol, 0.05% Tween-80 and 2 µg/ml of Mycobactin J (Allied Monitor). *M. paratuberculosis* $\Delta sigH$ culture was grown in the presence of 50 µg/ml

hygromycin. For bacterial CFU counts, *M. paratuberculosis* strains were cultured on Middlebrook 7H10 media with or without appropriate antibiotics. The *M. paratuberculosis* $\Delta sigH$ was generated using allelic exchange as detailed before by our group [27].

2.2. Vaccine preparation

M. paratuberculosis strains were grown to an optical density (OD) 600 nm of $\sim 1 (2 \times 10^8)$ CFU/ml and bacteria was collected by centrifugation at $3200 \times g$ for 10 min. Cells were washed with PBS twice and resuspended in PBS. The OD600 nm was determined and the cell suspensions were adjusted to $\sim 5 \times 10^8$ CFU/ml for vaccine strains. 1 mg/mL of QuilA (Desert King, CA, USA) was prepared in sterile PBS and filter sterilized.

2.3. Co-culture of *M. paratuberculosis* strains with QuilA

Both wild-type and $\Delta sigH$ mutant strains were co-cultured in the presence of 0.1mg/mL QuilA up to 15 days. At various time points, aliquots of bacterial cultures were plated by serial dilutions on 7H10 media with or without 50 μ g/ml of hygromycin to determine effect of QuilA on the survival of *M. paratuberculosis* strains.

2.4. Mice immunization.

Female C57/BL6 mice were purchased from Taconic (Hudson, NY, USA) and housed at the University of Wisconsin-Madison. All animal experiments used in this study were performed according to the protocols approved by the Institutional Animal Care and Use Committee. Various groups of mice ($n = 8-14$) were subcutaneously (s.c.) immunized into the neck scruff. A control group ($n = 12$) was immunized with PBS while *M. paratuberculosis* $\Delta sigH$ mice group ($n = 12$) received $\sim 2 \times 10^6$ CFU in 0.2 ml PBS, 2 weeks apart. Four weeks following booster dose, a subgroup of mice was challenged intraperitoneally (i.p.) with $\sim 5 \times 10^8$ CFU wild-type *M. paratuberculosis* K10 strain as determined by plate count on the day of infection (Fig. 1).

In a separate immunization scheme (Fig. 1), mice were vaccinated with one of the following formulations: mice groups ($n = 8$) were immunized once s.c. into the neck scruff with $\sim 10^8$ CFU live *M. paratuberculosis* $\Delta sigH$; heat-killed *M. paratuberculosis* strain; or live 10^8 CFU *M. paratuberculosis* $\Delta sigH$ containing QuilA (20 μ g) adjuvant. An additional mice group was vaccinated (50 μ l/mouse) with Mycopar™ (Boehringer Ingelheim, CT, USA) using s.c. route. Six weeks following vaccination, a subgroup of mice was challenged i.p. with a cattle type clinical isolate *M. paratuberculosis* JTC1285 ($\sim 10^9$ CFU/mouse). Mice groups ($n = 4-6$) were sacrificed and organ samples were collected at 6 weeks post initial immunization (WPI) and 6, 9 or 12 weeks post challenge (WPC) to evaluate the immune responses, histopathological examinations and bacterial tissue colonization levels as described before [26]. Liver and spleen tissue homogenates were serially diluted in PBS following plating on 7H10 media. For intestines, 7H10 plates were supplemented with 5 mg/ml vancomycin, 30 mg/ml amphotericin B, and 10 mg/ml nalidixic acid antibiotic mixture to reduce nonmycobacterial and fungal contamination. Aliquot of organ homogenates were plated on 7H10 media containing 50 μ g/ml of hygromycin to determine organ burden of the vaccine strain and whenever necessary, vaccine strain CFU counts were subtracted from the total *M. paratuberculosis* organ loads to determine actual level of organ colonization for challenge strain.

2.5. Evaluation of immune responses.

Mouse spleens were collected aseptically and homogenized by gentle mechanical disruption using a stainless steel screen. Following isolation of mononuclear spleen cells, the red blood cells were lysed using 0.83% ammonium chloride. Spleen cells were washed and suspended in RPMI 1640 (Thermo Fisher, PA, USA) supplemented with $1\times$ non-essential amino acids (Invitrogen), $1\times$ L-glutamine, $1\times$ Penicillin streptomycin, and 10% fetal bovine serum (Atlanta Biologicals,

GA, USA). Following trypan blue dye enumeration for cell viability, splenocytes were cultured in duplicate in round bottom 96-well tissue culture plates with 1×10^6 cells/well containing 100U/mL human IL-2 (BD biosciences, NJ, USA) [28]. Cells were re-stimulated in vitro with media alone or media supplemented with 10 µg/ml Johnin purified protein derivative (PPDj) (USDA–National Veterinary Services Laboratory, Ames, IA) or 10 µg/ml whole cell crude lysate (WCL) for 48 and 72 h at 37°C with 5% CO₂ [19,29]. Cell supernatants were collected and levels of secreted cytokines were analyzed by mouse cytokine ELISA kit according to the manufacturer's instructions (BioLegend, CA, USA). The level of cytokine secretion in the media stimulation was subtracted from the PPDj or WCL stimulated spleen cells.

2.6. Antibody IgG ELISA

Sera were harvested from whole blood collected at the same time as tissue collection to detect immunoglobulin G (IgG), IgG1 (Th2 marker), and IgG2a (Th1 marker) antibodies as previously described [19,30]. For ELISA detection of overall antibody response (IgG), 96-well high-binding Microton flat-bottom plates (Greiner Bio-One, NC, USA) were coated with 100 µl/well *M. paratuberculosis* whole cell lysate (5 µg/ml) and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked with PBS containing 1% bovine serum albumin (BSA) for 2 h at 37°C. After three washes with PBST, 100 µl of serum diluted 1:200 in PBS containing 0.75% BSA was added to the wells and incubated for 1 h at 37°C. After washing, 100 µl rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP) (1:10,000) was added, and plates were incubated for 1 h at 37°C. After three washes with PBST, 100 µl of tetramethylbenzidine (TMB) substrate was added to each well, and plates were incubated in the dark for 20 min. The enzymatic reaction was stopped

by the addition of 1 M H₂SO₄, and the optical density (O.D.) was read at 450 nm using a SpectraMax plate reader (Molecular Devices, CA, USA).

Serum IgG2a and IgG1 were evaluated using ELISA by coating 96-well high-binding Microton flat-bottom plates (Greiner Bio-One) with 1 µg/ml of coating antibody (anti-IgG2a or anti-IgG1) (Bethyl Laboratories Inc., TX, USA) diluted in 0.05 M carbonate/bicarbonate buffer (pH 9.6) for 1 h at room temperature. The plates were washed five times with washing buffer (50 mM Tris, 0.14 M NaCl, and 0.05% Tween 20) and blocked with blocking buffer (50 mM Tris, 0.14 M NaCl, and 1% BSA) overnight at 4°C. After washing, sera were diluted 1:5,000 in sample buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, and 0.05% Tween 20) and Mouse Reference Serum (Bethyl Laboratories Inc.) was diluted two-fold starting at a concentration of 1000 ng/ml for plotting the standard curve. 100 µl of each sample was placed in the appropriate wells and incubated for 1 h at 37°C. After washing, 100 µl HRP conjugated goat anti-mouse IgG2a or IgG1 detection antibody (1:75,000) was added to each well and incubated for 1 h at 37°C. After washing five times, 100 µl of TMB substrate was added to each well, and plates were incubated in the dark for 15 min. The reaction was stopped with 0.18 M H₂SO₄, and the optical density was measured at 450 nm.

2.7. Statistical analysis.

Differences between groups were analyzed with a one-way analysis of variance (ANOVA) or Student *t* test. For multiple comparisons, data points were compared by a Kruskal–Wallis test followed by Tukey and Dunn’s pair-wise comparison. A probability value of <0.05 was considered significant for all tests.

3. Results

3.1. Experimental design of the vaccine trials.

We have previously demonstrated the important role of a set of *M. paratuberculosis* gene regulators (e.g. sigma factors) during bacterial intracellular survival [19,26]. In addition, when *M. paratuberculosis* $\Delta sigL$ mutant was tested for its vaccine potential, protective immune responses were generated in a vaccine/challenge model of murine paratuberculosis [19]. These studies highlighted the critical role of sigma factors in the pathogenesis of *M. paratuberculosis* infection and demonstrated the viability of developing an effective control strategy against paratuberculosis in animals. Based on an earlier report [26] identifying sigma factor H (*sigH*) as virulence factor, we immunized groups of mice with *M. paratuberculosis* $\Delta sigH$ mutant alone or combined with a known adjuvant, such as QuilA, to boost the immunogenicity of the vaccine candidate. To better evaluate the performance of live-attenuated vaccine constructs, we immunized other groups of mice with heat-inactivated *M. paratuberculosis* or the available licensed vaccine for Johne's disease, Mycopar™. Mice groups were challenged with virulent strains of *M. paratuberculosis* (laboratory isolate K10 or field isolate JTC1285) where organ bacterial burdens were evaluated following challenge (Fig. 1). The genome structure for challenge strains were sequenced earlier [27,31] and confirmed to belong to *M. paratuberculosis* cow-type strains.

3.2. Stability and persistence of vaccine constructs

To examine stability of the LAV constructs in the presence of an adjuvant, we have cultured *M. paratuberculosis* strains in the presence of 0.1mg/mL QuilA in vitro. This concentration of QuilA was found to be safe and immunogenic in mice [32]. In general, growth patterns between the wild-type strain and *M. paratuberculosis* $\Delta sigH$ mutant were similar, suggesting ability of the vaccine strain to survive in the QuilA based adjuvant for an extended period of time (Fig. 2A). To examine persistence of the mutant strain in animals, groups of mice immunized with 10^6 CFU

M. paratuberculosis $\Delta sigH$ were analyzed for bacterial content at 6 weeks post vaccination and before any challenge. Two immunizations with this mutant resulted in low colonization ($\sim 6 \times 10^3$ CFU) in the spleen whereas no bacteria were detected (limit of detection 20 CFU) in the intestine or liver (data not shown). However, when mice groups were immunized with the live-attenuated vaccine strain, $\Delta sigH$, with a single, higher dose (10^8 CFU/mouse) (Fig. 2B), organs were colonized at lower levels in livers and spleens. However, $\Delta sigH$ mutant was not detectable when plated on 7H10 media containing 50 μ g/ml of hygromycin to determine organ burden of the vaccine strain at 9 or 12 WPC suggesting limited ability of the vaccine strain to survive inside the host following immunization. Histopathological analysis of all mice groups at 6 weeks post immunization did not reveal significant lesion scores, only the presence of minor lymphocytic infiltration in the livers of immunized animals. Overall, these experiments suggested the stability and persistence of the *M. paratuberculosis* $\Delta sigH$ mutant as LAV candidate in mice.

3.3. Immune responses generated by LAV.

To evaluate vaccine-induced recall responses to *M. paratuberculosis* whole cell lysate, spleens were harvested from mice 6 weeks post immunization and prior to any challenge in 2 different vaccine trials. The secreted IFN- γ and IL-10 levels were measured to evaluate *M. paratuberculosis*-specific cell mediated and humoral immune responses, respectively. All immunization protocols with LAV or inactivated constructs elicited significantly higher IFN- γ levels compared to PBS vaccinated mice (Fig. 3A). For IL-10, all groups elicited higher levels of IL-10 compared to PBS-control group (Fig. 3B) but only MycoparTM provided significant IL-10 levels compared to $\Delta sigH$ vaccinated mice. In a subsequent experiment, we used QuilA-adjuvant to improve the immunogenicity of the LAV ($\Delta sigH$) construct. Indeed, mice groups which received QuilA adjuvant vaccine secreted significant *M. paratuberculosis*-antigen-specific

IFN- γ responses compared to the *M. paratuberculosis* $\Delta sigH$ alone (Fig. 3C). However, such induction was not significant when IL-10 response was measured (Fig. 3D). Whether QuilA was included in the vaccine formula or not, higher levels of both IFN- γ and IL-10 were measured in comparison to the control group (immunized with QuilA only).

To further dissect the type of elicited immune responses following each immunization protocol, we evaluated the levels of serum antibodies. As expected, all of the vaccinated groups elicited significantly higher levels of *M. paratuberculosis*-specific serum IgG compared to respective control groups (PBS or QuilA; Fig. 4A, B). When IgG subtypes were analyzed, the IgG2a to IgG1 ratio displayed a higher level of IgG1 for all groups (Fig. 4C). When comparing subtypes between the groups, however, no statistically significant difference was shown in IgG1 (Th2 marker) between the groups. Subtyping data also showed that mice groups that received *M. paratuberculosis* $\Delta sigH$ with the QuilA adjuvant elicited slightly better IgG2a (Th1 marker) response, even though not significant, compared to mice group that received $\Delta sigH$ vaccine alone (Fig. 4D). Overall, our data indicated ability of the LAV strain to induce similar *M. paratuberculosis*-antigen-specific IFN- γ response compared to heat-killed and Mycopar™ vaccine constructs. In addition, the use of QuilA adjuvant was beneficial to enhance the elicited host cellular immunity.

3.4. Protection induced by $\Delta sigH$ LAV.

To analyze the protective immunity of vaccine constructs, immunized mice groups were challenged with the virulent *M. paratuberculosis* K10 strain at 6 WPI. After 6 weeks post challenge (WPC), the $\Delta sigH$ vaccinated-mice displayed a significant reduction in the bacterial load in intestine (>10 folds), an important organ for *M. paratuberculosis* infection, compared to

the PBS-vaccinated mice (Fig. 5). A similar significant reduction in bacterial load was observed at 12 WPC in all examined organs, e.g. intestine, liver and spleen.

To further evaluate the impact of adjuvant on LAV performance, animal groups were vaccinated with higher dose of the LAV $\sim 10^8$ CFU/mouse in presence or absence of QuilA and challenged with a clinical cattle type strain, *M. paratuberculosis* JTC 1285. At 9 WPC, $\Delta sigH$ LAV performed better compared to MycoparTM vaccine and provided significant reduction of bacterial load (>15 folds) in the liver and spleen (Fig. 6). Moreover, $\Delta sigH$ vaccinated-mice displayed >20 folds reduction in the bacterial load in intestine compared to the MycoparTM vaccinated group (Fig. 6C). Interestingly, in a few LAV vaccinated animals, no colonies were detectable in the intestines as suggested by our inability to culture any bacteria (limit of detection 20 CFU) at 9 WPC from this organ. However, we did not see any additional effect of QuilA adjuvant in reducing bacterial load as indicated by similar tissue colonization between mice groups whether QuilA adjuvant was used or not (Fig. 6).

Histologically, the sizes of the lymphocytic infiltrations were larger in mice of the MycoparTM vaccinated group while those found in the LAV groups were more defined and smaller in lesion size in the liver (Fig. 7 A-C) indicating that the $\Delta sigH$ LAV can reduce tissue damage from *M. paratuberculosis* infection. Notably, livers and spleens (data not shown) of LAV immunized groups showed low levels of lymphocytic infiltration associated with macrophage aggregates (scores range 2-3 out of 5) at 9 WPC (Fig. 7D). On the contrary, lesion scores for lymphocytic infiltrations ranged between 4-5 for the MycoparTM vaccinated group at the same time point (Fig. 7D).

Finally, to examine expansion of the cellular immune response following challenge, splenocytes of immunized mice were analyzed at 9 WPC. Both IFN- γ and IL-10 levels were similar among

vaccine groups (LAV or Mycopar™) when splenocytes were stimulated with *M. paratuberculosis* whole cell lysate (Fig. S1). Collectively, vaccination with the $\Delta sigH$ LAV constructs reduced bacterial burden and tissue pathology generated by the challenge with virulent strains of *M. paratuberculosis*.

4. Discussion.

The development of an effective vaccine to confer protection to dairy cattle against *M. paratuberculosis* infection is critical for control and prevention of Johne's disease. Previously, various immunization reports suggested positive effects of the whole cell inactivated vaccines including decreased fecal shedding following challenge with *M. paratuberculosis*, delayed onset of the clinical disease and increased average day milk production [8-11]. However, despite the proven usefulness in limiting disease progression, the whole cell inactivated vaccines have limited ability to prevent new infection [33]. Recently, several studies targeted live attenuated mutant strains of *M. paratuberculosis* towards developing a better vaccine against Johne's disease, even though the development of new live vaccine candidates is still in its infancy [20,21,34]. Live attenuated vaccines have been considered as an effective alternative strategy to control *M. paratuberculosis* infection because of their safety and ability to induce host immunity that limits bacterial organ colonization and provides protection against *M. paratuberculosis* infection [19,20,22,24]. In this report, we used a rational approach to investigate a virulence-attenuated mutant strain for vaccine development against this chronic illness. We also evaluated the importance of using adjuvants and compared live-attenuated vs. inactivated vaccine constructs.

Earlier, we have established that the alternative sigma factor, *sigH*, is a global gene regulator (GGR) controlling a large number of genes in *M. paratuberculosis* [26]. Gene deletion in this sigma factor showed both decreased survival in primary bovine macrophages and

virulence-attenuated phenotype in mouse infection model for this isogenic knock-out mutant, $\Delta sigH$, when compared to the wild-type strain. Based on these results, we hypothesized that $\Delta sigH$ mutant strain would undergo limited replication inside the host following vaccination and thus, be immunogenic eliciting a long-lasting protective immunity. To test this hypothesis, we examined this live-attenuated strain in a vaccine/challenge model of murine paratuberculosis. However, we acknowledge the limitations associated with the murine infection model as mice are not the natural host and they do not exhibit standard clinical signs (e.g. diarrhea) following successful infection. Nonetheless, this is a good model for initial assessment (e.g. immunologic parameter, bacterial organ burden levels) of anti-*M. paratuberculosis* vaccines along with further validation of immune correlates of protection in ruminant models (e.g. goat and calf) could lead to an improved vaccine desperately needed to control Johne's disease.

Immunological analyses conducted here indicated that the GGR based *M. paratuberculosis* mutant elicited strong immune responses such as IFN- γ , an important cytokine involved in controlling mycobacterial infection [35], suggesting their immunogenicity and ability to potentially initiate effective vaccine-induced immunity [19]. In addition, antibody subtyping also demonstrated the capacity of live vaccine candidates to induce strong serum antibody response and that major IgG isotypes expressed within the vaccinated animals predominantly was IgG1 with modest IgG2a level. The role of humoral immunity in *M. paratuberculosis* infection is not completely understood. The immune status of the animals suffering from Johne's disease suggests a shift to the humoral Th2 response from the protective Th1 cellular immune response indicating humoral immunity is not protective during clinical state of *M. paratuberculosis* infection [36,37]. However, recent reports demonstrated that humoral immunity could play an important role against *M. tuberculosis* infection [38-40]. In general, our data also

demonstrated safety and immunogenicity of this vaccine strain as shown by a low-level of bacterial tissue burden at 6 weeks post vaccination. More importantly, we found that the vaccine conferred protective immunity as indicated by the reduced bacterial colonization in all examined tissues following challenge with a virulent strain of *M. paratuberculosis*. Previously, the alternative sigma factor (e.g. *sigL*) mutants were targeted for the vaccine development and the isogenic knock-out mutant strains were found to be immunogenic providing protection against infection with *M. paratuberculosis* and *M. tuberculosis* [19,41,42]. Earlier studies with the $\Delta leuD$ strain, an *M. paratuberculosis* mutant defective in leucine biosynthesis, demonstrated better protection using a similar vaccine/challenge model [43]. However, the methodology employed in $\Delta leuD$ study makes it difficult to directly compare $\Delta leuD$ mutant with the GGR based mutant strain analyzed in the current study because tissue colonization levels in the *LeuD* experiments were analyzed using only Ziehl-Neelsen staining [44,45], instead of colony counting used in our study. Our data analyses suggested that both $\Delta sigH$ and $\Delta sigL$ LAV conferred similar protection levels following challenge with the virulent *M. paratuberculosis* strain at 6 weeks [19]. Interestingly, $\Delta sigL$ vaccinated mice displayed better reduction in the bacterial load in liver compared to the mice group that received $\Delta sigH$ vaccine [19]. Overall, these findings demonstrated a “proof of principal” study that GGR based virulence-attenuated *M. paratuberculosis* mutant strains are effective live-attenuated vaccines against paratuberculosis.

Next we compared performance of the GGR based live attenuated vaccine against the currently available licensed vaccine, Mycopar™, against paratuberculosis in USA. Additionally, we evaluated efficacy of the vaccine in the presence of QuilA based adjuvants that has been approved for use in veterinary medicine [46-49]. Our data indicated that $\Delta sigH$ conferred better protective immunity compared to the Mycopar™ group as suggested by their ability to induce

host immunity that was sufficient to reduce tissue colonization and lesion scores in animals following challenge with a cattle type clinical strain of *M. paratuberculosis*. Moreover, our data demonstrated beneficial effect of QuilA adjuvant in vaccination as indicated by enhanced host cellular immunity. However, we did not see any significant differences in bacterial colonization levels following challenge with *M. paratuberculosis* between mice groups that received vaccine with or without QuilA adjuvant. Interestingly, recent studies showed that a double gene deletion mutant constructed in *M. tuberculosis*, $\Delta fbpA\Delta sapM$, was highly attenuated in human macrophages and more immunogenic than either $\Delta fbpA$ or $\Delta sapM$ suggesting potential of the double mutant strain as anti-tuberculosis vaccine [50]. In future experiments, it would be helpful to generate a double knockout vaccine candidate with a deletion of the *sigH* gene in the $\Delta sigL$ mutant, a LAV previously shown to be immunogenic providing protection following challenge with *M. paratuberculosis* [51], to increase vaccine safety and immunogenicity as well as avoid risk for vaccine reversion.

In summary, our data showed that the $\Delta sigH$ live vaccine strain was highly immunogenic and performed better compared to the Mycopar™ vaccine. Further vaccine testing in natural hosts of Johne's disease, e.g. goats, will demonstrate the viability of developing an effective control strategy against paratuberculosis in ruminants. Our approach also provides a rational basis for using global gene regulator based live attenuated vaccines to develop an improved vaccine that elicits protective immunity without the risk of a persistent *M. paratuberculosis* infection, at least in mice model. We are confident that such approach will not only strengthen our knowledge in *M. paratuberculosis* pathogenesis but also contribute to the development of vaccines urgently required as a control measure for paratuberculosis.

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Figure legends.

Fig. 1. Schematic outline for the vaccine/challenge trials. Challenge studies illustrates infection with the wild-type *M. paratuberculosis* K10 (A) or a clinical strain, *M. paratuberculosis* JTC1285 (B). In scheme A, control group ($n = 12$) was immunized s.c. with PBS while *M. paratuberculosis* $\Delta sigH$ mice group ($n = 12$) received $\sim 2 \times 10^6$ CFU in 0.2 ml PBS, 2 weeks apart. Four weeks following booster dose (week 0), mice groups were challenged i.p. with $\sim 5 \times 10^8$ CFU wild-type *M. paratuberculosis* K10 strain. In scheme B, mice ($n = 8$) groups were vaccinated with PBS; QuilA (20 μ g/mouse); $\sim 10^8$ CFU live *M. paratuberculosis* $\Delta sigH$; heat-killed *M. paratuberculosis* strain; live 10^8 CFU *M. paratuberculosis* $\Delta sigH$ containing QuilA adjuvant; or Mycopar™ (50 μ l/mouse). Six weeks following vaccination (week 0), mice were challenged i.p. with a cattle type clinical isolate *M. paratuberculosis* JTC1285 ($\sim 10^9$ CFU/mouse). Mice groups ($n = 4-6$) were sacrificed and organ samples were collected at 6 weeks post initial immunization (WPI) and 6, 9 or 12 weeks post challenge (WPC) to evaluate the immune responses, histopathological examinations and bacterial tissue colonization.

Fig. 2. In vitro and in vivo survival of $\Delta sigH$ mutant. (A) Sustained effect of exposure to QuilA on *M. paratuberculosis* survival. Both wild-type and $\Delta sigH$ mutant strains were cultured in the presence of 0.1mg/mL QuilA up to 15 days. Error bars represent the standard deviations calculated from the means of colony counts estimated from two independent experiments. (B) Survival of $\Delta sigH$ mutant in mice at 6 WPI. Organ homogenates e.g. liver and spleen were plated on 7H10 + ADC with hygromycin (50 μ g/mL) to examine survivability of $\Delta sigH$ mutant. Detectable bacterial colonies indicated ability of the mutant strain to survive in the vaccinated animal tissues. Horizontal lines indicate median value for each group.

Fig. 3. Analysis of immune responses in immunized mice before challenge. Following vaccination and before challenge, mice (N = 4-6) from each group were sacrificed for analysis of immune response after 6 weeks post immunization (6PWI; week 0 in Fig. 1). Splenocytes were isolated and re-stimulated in vitro with whole cell lysate to measure IFN- γ (A) and (B) IL-10 levels from culture supernatant by ELISA after 48 h. (C-D) Effect of QuilA adjuvant on host immunity. Mice groups were vaccinated with $\Delta sigH$ LAV with or without QuilA (20 μ g). All groups were significant when compared to PBS or QuilA group. * p <0.05, when each indicated group was compared to $\Delta sigH$ vaccine group.

Fig. 4. *M. paratuberculosis* specific antibody response. Mice sera isolated following vaccination and before challenge were analyzed for *M. paratuberculosis* whole cell lysate-specific antibodies. Levels of total IgG (A, B) and subtypes (C, D) IgG1 and IgG2a were determined using a *M. paratuberculosis* antibody ELISA. * p <0.05, when each indicated group was compared to PBS or QuilA group; † p <0.05, when each indicated group was compared to $\Delta sigH$ vaccine group; # p <0.05, $\Delta sigH$ versus Mycopar™.

Fig. 5. Protective efficacy of LAV immunization. 6 weeks following vaccination, mice received a challenge dose containing $\sim 5 \times 10^8$ CFU wild-type *M. paratuberculosis* K10 strain i.p (see Fig. 1A). Following challenge, mice groups (N=4) were sacrificed at 6 and 12 weeks and bacterial burden was analyzed in intestine (A), liver (B), and spleen (C) organs. Horizontal lines indicate median value with error bars representing the standard deviation. Statistical analyses

were done using Mann-Whitney test to evaluate differences in bacterial organ load among mice groups vaccinated with the PBS or *M. paratuberculosis* $\Delta sigH$ mutant. * $p < 0.05$

Fig. 6. LAV provided better protection compared to Mycopar™. 6 weeks following vaccination, mice received a challenge dose containing $\sim 5 \times 10^8$ CFU *M. paratuberculosis* JTC1285 clinical strain i.p (see Fig. 1B). Following challenge, mice groups (N=4) were sacrificed at 9 weeks and bacterial burden was analyzed in liver (A), spleen (B), and intestine (C) organs. Horizontal lines indicate median value with error bars representing the standard deviation. The limit of detection (20 CFU) in the intestine tissues is indicated by a dotted line. Statistical analyses were done using ANOVA to evaluate differences in bacterial organ load among mice groups vaccinated with the Mycopar™ or $\Delta sigH$ LAV strain. * $p < 0.05$

Fig. 7. Liver histopathology of mice groups after challenge with *M. paratuberculosis* JC1285. Liver sections stained with hematoxylin and eosin (100× magnification; scale bar = 200 μ m) collected from (A) Mycopar, (B) $\Delta sigH$, and (C) QuilA+ $\Delta sigH$ vaccinated mice groups at 9 weeks post challenge (9 WPC). Arrows indicate granulomas infiltrates (GI). A representative GI is shown in the inset picture (white arrow). (D) Liver inflammatory histological scores. Each dot represents the score given to an individual mouse. Score of 1-mild, 2-notable, 3-severe, 4-severe with spread, and 5-severe with very widespread. Significance was determined by Kruskal–Wallis test followed by a Dunn’s pair-wise comparison. * $p < 0.05$.

Fig. S1. Analysis of immune responses in immunized mice after challenge. Following vaccination and 9 weeks post challenge, mice (N = 4-6) from each group were sacrificed for

645 analysis of immune response. Splenocytes were isolated and re-stimulated in vitro with whole
646 cell lysate to measure IFN- γ (A) and (B) IL-10 levels from culture supernatant by ELISA after
647 48 h.

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