Randomized controlled trial examining the immunogenicity of a *Salmonella* Dublin siderophore receptor vaccine in Holstein calves

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Introduction

Salmonella Dublin is a common cause of calf illness, with high morbidity and mortality rates in affected animals, and is endemic in many regions of the U.S. and Canadian dairy industries.[1–3] A 2016 report[4] from the U.S. National Veterinary Services Laboratory found that *S.* Dublin was the most isolated Salmonella serotype obtained from ill cattle in the U.S. This serotype is also considered to be host adapted in bovine. Therefore, carrier animals that appear normal can be intermittent shedders and a source for maintaining the infection within a herd.[3]

Finding mechanisms to protect naïve animals from clinical disease caused by *S.* Dublin can reduce losses of animals and improve animal welfare in dairy herds. This includes maximizing transfer of passive immunity, maintaining high standards of hygiene, removing calves from the maternity environment as quickly as possible to reduce exposure of *S.* Dublin bacteria shed by carrier dams, and effective vaccination of dams and calves early in life.[3]

Effective vaccination against Salmonella requires the production of both humoral (i.e. antibodies) and cell mediated (i.e. T lymphocytes) immune responses as Salmonella organisms can survive within macrophages.[5–8] To eliminate intracellular Salmonella, macrophages must become activated via the cytokine, interferon-γ (IFN-γ) which is released from T-helper (Th)-1 lymphocytes. Activated macrophages can more efficiently kill intracellular Salmonella through enhanced phagocytic activity and upregulated reactive oxygen species (ROS) and nitrous oxide (NO) synthesis.

Interleukin-17 is a cytokine that is produced by Th-17 cells and plays a key role during Salmonella infections by triggering inflammation and recruiting and activating inflammatory cells like neutrophils to the gut early in the course of disease as well as promoting the upregulation of antimicrobial molecules by neutrophils and gut epithelial cells.[5]

Traditional killed vaccines have failed to stimulate effective cell-mediated immune responses,[9–11] so alternative vaccine technologies are needed. A possible strategy to induce effective immunity against *S.* Dublin is to target its iron-acquisition system. Iron is an essential nutrient of all gram-negative bacteria. In low-iron environments, such as in mammalian tissues, Salmonella manufacture and excrete low molecular weight proteins with a high affinity for iron called siderophores. Siderophores bind iron forming siderophore-iron complexes. Siderophore receptor proteins (SRP®) are receptors located in the outer membrane of Salmonella bacteria and are responsible for transporting siderophore-iron complexes into the bacterial cell for use. Vaccines that utilize SRP® from *S.* Dublin should restrict the transportation of iron into the bacterium, thereby starving it of this required nutrient, resulting in cell death. Siderophore receptor proteins are highly conserved among gram-negative bacteria and are nearly identical within a genus, making them novel vaccine targets.

Objective

The objective of this study was to investigate the immune responses stimulated by a *S.* Dublin SRP® vaccine in Holstein heifer calves.

Materials and Methods

This study was conducted on a commercial dairy farm with no history of disease associated with *S.* Dublin and not using any Salmonella or SRP® vaccines in adult animals or calves. The infection status of the herd was verified by their veterinarian, who had multiple years of experience with the operation.

To confirm *S.* Dublin–negative status of the lactating herd, bulk tank milk was collected four times throughout the study at approximately 3-week intervals and 1 and 2 months after the study was completed. The milk samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory and tested via a commercial Salmonella ELISA (PrioCHECK). Additionally, all trial calves were checked for infection status via the same ELISA kit on serum collected between 90 and 120 days of age.

At approximately 7 days of age (range: 4 – 10 days of age) and 3 weeks later, Holstein calves were randomized to receive 1 ml subcutaneously in the neck of either 1) experimental *Salmonella* Dublin SRP® vaccine (**VACCINE**), or 2) sterile saline (**PLACEBO)**. Only calves that were healthy and had a serum total protein value ≥ 5.5 mg/dL between 1 and 7 days of age were vaccinated at each time point. Farm staff was trained to watch calves on the days of vaccination and to notify trial personnel if they noticed any problems.

Prior to each vaccination, at 4 and 8 days after the second vaccination, and 61 to 91 days after the second vaccination (approximately 90 to 120 days of age), serum was collected for antibody (Ab) titer analysis using an ELISA. Additionally, at 4 and 8 days after the second vaccination, whole blood was collected for isolation of peripheral blood mononuclear cells (PBMC) for cell-mediated immunity analyses. Specifically, bovine IFN-γ assays (ELISpot) were used to quantify the number of SRP®-responsive Th-1 lymphocytes. Briefly, PBMC were resuspended in cell medium, plated in duplicate ELISpot wells, and stimulated with *S.* Dublin SRP® antigen. Plates were read on an ELISpot reader and results were reported as spot-forming units/10^6 stimulated cells. In parallel cultures, PBMC were also stimulated with *S.* Dublin SRP® antigens with supernatants used to quantify concentrations of the cytokines, IFN-γ and IL-17 in duplicate per the manufacturer's instructions.

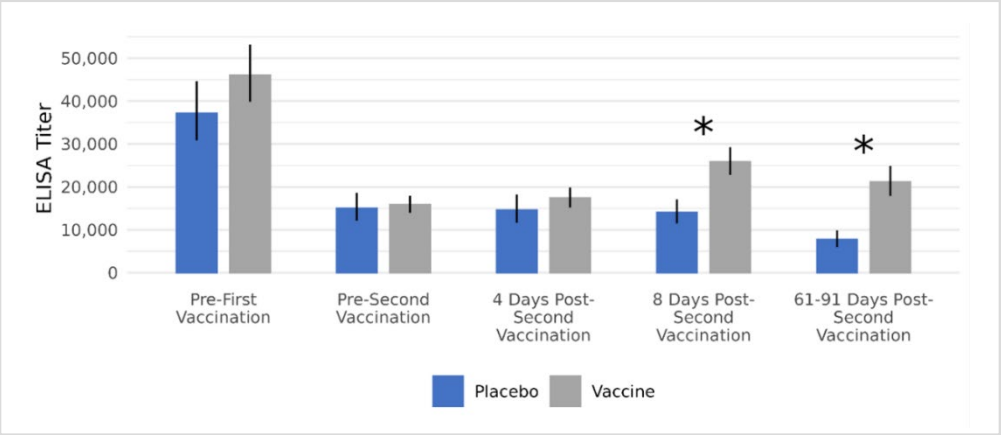
Statistical analysis was completed using freely available statistical software (R, version 4.3.0; R Foundation for Statistical Computing) with an added package for streamlining and plotting (Tidyverse, version 2.0.0). Descriptive statistics were compared using ANOVA for the numeric variables and chi-square or proportion tests for the health event data. Enzyme-linked immunosorbent assay titers and cell-mediated immune responses for placebos and vaccinates were compared at each time point independently to reduce the compounding impact of developmental shifts in immune response. Log transformation was used for all titer analyses. The results for humoral and cell-mediated data are presented as the geometric mean of each group.

Results and Discussion

A total of 78 Holstein heifer calves were enrolled in this study with 54 vaccinates and 24 controls. No adverse reactions other than occasional minor swellings at the injection site were reported following vaccination. All bulk tank milk and individual serum Salmonella ELISA tests were negative.

Figure 1 shows the geometric mean of Ab titers for placebo and vaccine groups throughout the study. Prior to each vaccination and at 4 days post second vaccination, no significant differences in Ab titers were detected between groups. At 8 days post second vaccination, Ab titers were significantly higher ($P < 0.001$) in vaccinates (mean Ab titer: 25,811) compared to placebo-treated calves (mean Ab titer: 14,011), with a difference of 11,800. A difference of 13,446 persisted at the final blood collection time point of 61 to 91 days post second vaccination (approximately 90 to 120 days of age; $P < 0.001$).

Figure 1. Geometric mean antibody titers for calves before their first and second vaccinations, at 4 and 8 days after the second vaccination, and 61 to 91 days after the second vaccination with either a *S.* Dublin SRP® vaccine or saline placebo. Time points marked with an asterisk are significantly different ($P < 0.001$).



The highest Ab titers were noted prior to the first vaccination, where placebo calves had a mean titer of 37,099 and vaccinated calves had a mean titer of 46,049. These high initial titers were likely from the passive transfer of maternal Ab developed against porin proteins from environmental gram-negative bacteria.

While there was no evidence of a vaccine effect on Ab titers at the time of the second vaccination, there was rapid increase in titer resulting in a significant difference between groups by 8 days after the second vaccination. These data demonstrate that a substantial anamnestic response did occur following the second dose of vaccine which was maintained through 90 – 120 days of age. While it is not known what titer is sufficient for prevention of disease caused by *S.* Dublin, these data demonstrate that this *S.* Dublin SRP® vaccine stimulates an increased Ab titer in comparison to the placebo when administered to calves at 1 and 4 weeks of age.

Figure 2. Geometric mean interferon-γ ELISpot results at 4 and 8 days after second vaccination with either a *S.* Dublin SRP® vaccine or saline placebo. Time points marked with an asterisk are significantly different (P < 0.001).

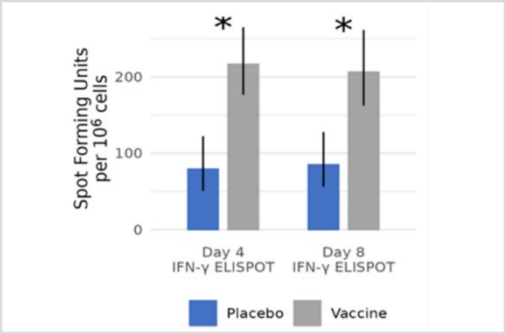


Figure 2 shows the geometric mean IFN-γ ELISpot results after stimulation of PBMC with *S.* Dublin SRP® antigen collected 4 and 8 days after second vaccination with either *S.* Dublin SRP® vaccine or saline placebo. ELISpot data represents the number of antigen-specific Th-1 lymphocytes secreting the cytokine, IFN-γ. Vaccinates had significantly higher numbers of Th-1 lymphocytes secreting IFN-γ at 4 (P < 0.001) and 8 days (P < 0.001) after the second vaccination compared to placebos.

Figure 3. Geometric mean IL-17 and IFN-γ concentrations at 4 and 8 days after the second vaccination with either a *S.* Dublin SRP® vaccine or saline placebo. Time points marked with an asterisk are significantly different (P ≤ 0.036).

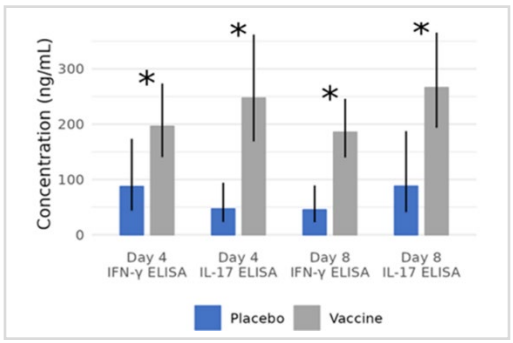


Figure 3 shows the geometric mean concentrations of the cytokines, IL-17 and IFN-γ following stimulation of PBMC with *S.* Dublin SRP® antigen collected 4 and 8 days after second vaccination with either *S.* Dublin SRP® vaccine or saline placebo. Using ELISA, a significant difference was detected between vaccinates and placebos for IFN-γ and IL-17 concentrations at both time points. A difference of 109.0 (P = 0.036) and 140.5 (P < 0.001) ng/mL was determined for IFN-γ concentrations at 4 and 8 days after the second vaccination, respectively. For IL-17, a difference of 200.5 (P < 0.001) and 178.3 (P = 0.009) ng/ml was determined at 4 and 8 days after the second vaccination, respectively.

Studies in other species have demonstrated that both Th-1 lymphocyte and Th-17 responses are vital for the clearance of Salmonella bacteria.[12-14] Th-1 lymphocytes release IFN-γ which activates macrophages and allows them to more efficiently kill intracellular Salmonella through enhanced phagocytic activity and upregulated generation of ROS and NO.[5,8] Interleukin-17 plays a key role in infections caused by Salmonella by triggering inflammation and recruiting and activating inflammatory cells like neutrophils to the gastrointestinal tract early in the course of disease as well as upregulating antimicrobial molecules by neutrophils and intestinal epithelial cells.[5] Our data show that young calves can generate *S.* Dublin SRP®-specific Th-1- and Th-17-type cellular immune responses, which are key factors for protection from systemic Salmonella infection.[15]

Summary

Vaccination with this *S.* Dublin SRP® vaccine stimulated statistically significant cell mediated and humoral immunity in Holstein calves compared to placebos when given at 1 and 4 weeks of age. Both cell mediated and humoral immune responses are known to be important in protecting cattle against Salmonellosis. Use of this *S.* Dublin SRP® vaccine should be considered, along with other on-farm management and sanitation interventions, to help control this highly virulent pathogen on dairy operations.

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