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Immune memory induced by intranasal vaccination with a modified-live viral vaccine delivered to colostrum fed neonatal calves

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ABSTRACT

Bovine respiratory disease (BRD) remains a major health problem despite extensive use of vaccines during the post-weaning period. Apparent vaccine failure is attributed, in part, to primary vaccination during the period of greatest risk for BRD, providing inadequate time for onset of protective immunity. The current study investigated whether intranasal (IN) vaccination of 3–6 week old calves with a modified-live viral (MLV) vaccine induced sufficient immune memory to prevent respiratory disease and accelerate onset of protective immunity 5 months later. Vaccine groups included naïve controls, a single IN vaccination at 3–6 weeks of age, primary IN vaccination at 6 months, and either an IN or subcutaneous (SC) booster vaccination at 6 months (n = 10/group). All calves were challenged with BHV-1 four days after vaccination at 6 months of age. Primary IN vaccination at 6 months did not significantly reduce clinical disease but significantly ($P < 0.01$) reduced virus shedding. A single IN vaccination at 3–6 weeks of age significantly ($P < 0.05$) reduced weight loss but did not reduce fever or virus shedding. Both IN and SC booster vaccinations, significantly ($P < 0.01$) reduced clinical disease but virus shedding was significantly ($P < 0.001$) reduced only by IN booster vaccination. Reduction in virus shedding was significantly ($P < 0.01$) greater following booster versus primary IN vaccination at 6 months. All vaccination regimes significantly ($P < 0.01$) reduced secondary bacterial pneumonia and altered interferon responses relative to naïve controls. Only IN booster vaccination significantly ($P < 0.05$) increased BHV-1 specific IgA in nasal secretions. These results confirm primary MLV IN vaccination at 3 to 6 weeks of age, when virus neutralizing maternal antibody was present, induced immune memory with a 5 month duration. This immune memory supported rapid onset of protective immunity four days after an IN booster vaccination.

1. Introduction

Primary viral respiratory infections are an important factor contributing to the incidence and severity of secondary bacterial respiratory infections in humans [1,2] and cattle [3,4]. Multiple viral pathogens are associated with the viral-bacterial synergy that results in fatal bovine respiratory disease (BRD) and bovine herpesvirus-1 (BHV-1) contributes to secondary bacterial infections caused by *Mannheima haemolytica* [5] and *Mycoplasma bovis* [4]. BRD remains the major cause of infectious disease and economic losses in the US cattle industry and often occurs in 5 to 6 month old calves, soon after they are weaned, transported, and commingled in large groups within feedlots [6,7,8].

BRD remains the primary cause of disease in 5 to 6 month old calves despite frequent use of vaccines for both viral and bacterial respiratory pathogens [7]. The apparent failure of vaccines to control BRD may be due, in part, to current vaccination practices. Many calves receive their first vaccination for BRD pathogens on arrival in the feedlot, which coincides with the period of highest risk for respiratory infections [9,10]. Commingling calves increases exposure to multiple BRD pathogens and there is limited time for vaccines to induce protective immunity. Further, most vaccines currently licensed for BRD are injected parenterally, either subcutaneous (SC) or intramuscular (IM), whereas viruses causing BRD are often inhaled and infect both the upper and lower respiratory tract. Systemic immune responses induced by parenteral MLV

Abbreviations: BHV-1, bovine herpesvirus-1; BRD, bovine respiratory disease; IFN, interferon; MLV vaccine, modified-live viral vaccine; URT, upper respiratory tract.

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vaccines have limited capacity to prevent BHV-1 infection in the upper respiratory tract (URT) following a primary vaccination 5 days prior to viral challenge [11].

Vaccination of young calves is one strategy proposed for inducing protective immunity during the post-weaning period. Passively transferred maternal antibody can, however, interfere with vaccination of young calves when using parenteral vaccines. Ellis et al. (2001) reported parenteral vaccination with a MLV bovine viral diarrhea virus (BVDV) vaccine had no significant impact on decay of BVDV2-specific maternal antibody and there was no evidence of immune memory following a booster vaccination [12]. This conclusion was challenged by Endsley et al (2004) who reported activation of BVDV2-specific T cells circulating in blood following intranasal (IN) injection of a live virus in young calves when BVDV2 neutralizing maternal antibody was present [13]. This study confirmed BVDV2-specific T cells persisted for 21 to 32 weeks after mucosal exposure to virus and a subsequent study confirmed T-cell memory was protective after maternal BVDV2-specific antibody declined to an undetectable level [14]. Therefore, IN delivery of virus was an effective immunization route to circumvent maternal antibody interference with the induction of effector and memory T cell responses.

More recent studies confirm IN vaccination of neonatal calves with a MLV vaccine provides an effective strategy to avoid maternal antibody interference and induce local IgA antibody responses in the URT [15]. The duration of specific IgA responses in this study was relatively brief but anamnestic IgA antibody responses occurred following a booster IN vaccination 5 weeks later. These observations are consistent with a previous study confirming the mucosal immune system is functional in newborn calves and a single IN MLV vaccine could induce protective immune responses when calves were vaccinated in the absence of maternal antibody [16]. There are concerns, however, regarding the duration of protective immune responses following IN vaccination of newborn calves with MLV vaccines. Ridpath et al. (2003) demonstrated protective immune memory to BVDV2 persisted for 7–9 months following IN injection of live virus in neonatal calves that had received BVDV2 neutralizing maternal antibody [14]. In contrast, Ellis et al. (2013) reported protective immunity for bovine respiratory syncytial virus (BRSV) waned between 9 and 14 weeks following a single IN vaccination of neonatal calves that had received maternal antibody [17]. These differences in immune memory may reflect differences in the immunogenicity of individual viral components included in multivalent MLV vaccines, with BVDV being more immunogenic than BRSV and BHV-1 [18].

Therefore, while IN vaccination may be an effective strategy to avoid vaccine interference by maternal antibody there remain concerns that duration of effector responses and immune memory may vary among individual viral components within multivalent MLV vaccines. To address concerns regarding the duration of mucosal immune memory for BHV-1 following IN vaccination we designed a vaccine study to investigate immune memory following IN vaccination of neonatal calves (less than 6 weeks old) with a MLV vaccine that included a BHV-1 component. Calves received maternal antibody through colostrum from dams previously vaccinated with the same multivalent MLV vaccine and transfer of neutralizing maternal antibody was confirmed at the time of vaccination. The presence of immune memory was evaluated 5 months later when calves were challenged with BHV-1 four days after either a primary or booster IN vaccination. Innate and acquired immune responses, virus shedding, and clinical disease were parameters used to assess immune memory. The presence of immune memory was determined by comparing cohorts of animals that received either no prior vaccination, a single IN vaccination as neonates, a single IN vaccination four days prior to viral challenge, or a primary IN vaccination as neonates and a booster IN vaccination four days prior to viral challenge. The potential to boost mucosal immune memory by a parenteral SC booster vaccination was also evaluated. These studies confirmed a single IN vaccination of neonatal calves induced immune memory that supported rapid onset of protective immunity able to significantly reduce both BHV-1 infection and clinical disease within four days following an IN but not SC booster vaccination.

2. Material and methods

2.1. Study design and experimental groups

Calves were purchased from a single herd consisting of 750 Angus-cross cows. Cows were vaccinated annually, 6–8 weeks prior to breeding, with a multivalent, modified-live viral (MLV) vaccine that contained BVDV1, BVDV2, BHV-1, BRSV and parainfluenza virus-3 (PI3). The herd was maintained through selection of replacement heifers from within the herd and skin biopsies from replacement heifers were tested for bovine viral diarrhea virus (BVDV) type 1 and 2 using PCR analysis of 5 pooled skin samples (Prairie Diagnostic Services Inc., Saskatoon, SK Canada). A total of 120–150 heifer calves and an additional 80 to 120 calves for use in clinical trial were tested annually for BVDV and no persistently infected (PI) calves were detected in the 4 years prior to the current study. All calves in the current study skin tested negative for BVDV. For the current study, calves were selected from multiparous cows vaccinated SC with a multivalent MLV vaccine (Vista 5 SQ, Merck Animal Health, Madison, NJ) 6–8 weeks prior to breeding the year prior to the current study.

Suckling, male and female Angus-cross calves were 3–6 weeks of age when randomly assigned to two treatment groups (n = 45/group; Fig. 1) and each calf was identified with a unique ear tag number. One group of calves (Saline) received an IN injection of 2 ml sterile, endotoxin-free saline (Lab Technologies Ltd, Paisley, UK). The second group (MLV vaccine) received a 2 ml dose of the multivalent MLV vaccine IN (Vista5 SQ; Serial 90230034-01972903). The two groups of cow-calf pairs were held in separate pastures for two weeks post-vaccination to prevent vaccine virus transmission to the Saline group. Calves in both treatment groups that were seronegative (VN <1:2) for BHV-1 at 5-6 months of age were selected for the second phase of the clinical trial. The second phase of the trial was performed following abrupt removal of calves from their dams and transportation 400 km to the research facility on the same day. After arrival, calves had ad libitum access to hay (mixed brome and alfalfa) and their diet was supplemented with 1.5 kg rolled oats/animal/day.

Based on serum samples collected 4.5 months after primary vaccination, 20 BHV-1 seronegative (VN titre <1:2) calves in the Saline group were randomly assigned to two treatment groups (n = 10/group). One group received a second IN saline injection (Group A: Saline + Saline) and the other group received a primary IN injection of MLV vaccine (Group D: Saline + MLV vaccine; Vista5 SQ; Serial 90230034-01972903) (Fig. 1). Thirty (30) BHV-1 seronegative (VN titre <1:2) calves from the MLV vaccine group were randomly assigned to three treatment groups (n = 10/group). One group received an IN saline injection (Group B: MLV vaccine + Saline). The second group received an IN booster with the MLV vaccine (Group C: MLV vaccine + MLV vaccine), and the third group received a SC booster with the MLV vaccine (Group E: MLV vaccine + SC-MLV vaccine) (Fig. 1). Vaccination was performed the day after calves were weaned and transported to the research facility (Day −4) and four days later all calves were aerosol challenged (Day 0) with virulent BHV-1 (108 isolate; 5×10^6 plaque forming units/calf) (Fig. 1). All procedures throughout the trial were conducted in accordance with guidelines approved by the Canadian

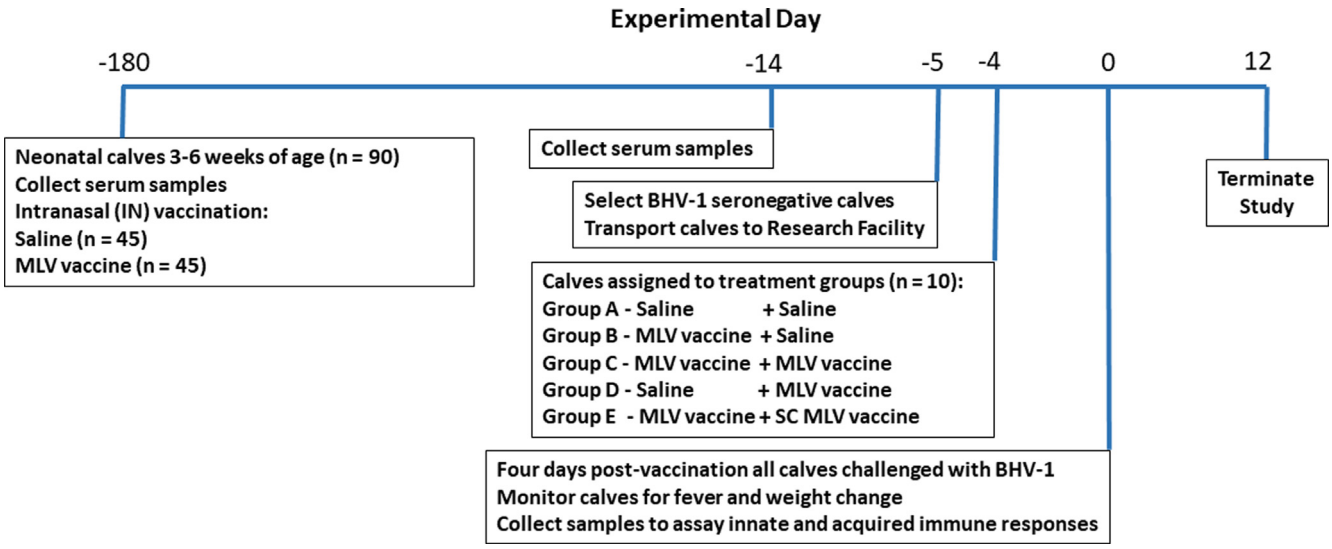

Fig. 1. Study design for analyzing immune memory following intranasal (IN) vaccination of neonatal calves with a modified-live viral (MLV) vaccine. Calves were vaccinated IN at 3–6 weeks of age with a modified-live viral vaccine (MLV vaccine) or received an IN saline injection (Saline). After 4.5 months, BHV-1 seronegative (VN titre <1:2) calves in the Saline group were randomly assigned to two groups (n = 10/group) that received either a second IN injection of saline (Group A: Saline + Saline) or a primary IN injection of the MLV vaccine (Group D: Saline + MLV). BHV-1 seronegative (VN titre < 1:2) calves from the MLV vaccine group were randomly assigned to three treatment groups (n = 10/group) receiving either an IN injection of saline (Group B: MLV vaccine + Saline), an IN booster vaccination (Group C: MLV vaccine + MLV vaccine), or a subcutaneous (SC) booster vaccination (Group E: MLV vaccine + SC MLV). All calves were aerosol challenged with BHV-1 four days later and then monitored for signs of clinical disease and innate and acquired immune responses for 12 days post-infection.

Council on Animal Care and endorsed by the University of Saskatchewan Animal Care Committee (Protocol 19940212 for vaccination; Protocol 19940218 for viral challenge; Protocol 20010015 for collection of blood and nasal secretions).

2.2. Serum collection and virus neutralization (VN)

Serum samples were collected and assayed for VN titres when 3–6 week old calves were first vaccinated and then 4.5 months later (Fig. 1). Serum VN titres specific for the viruses represented in the MLV vaccine were assayed by Prairie Diagnostic Services Inc (Saskatoon, SK Canada) [19]. The VN assay used the Singer strain and the 125 strain for testing BVDV 1 and 2 titres, respectively [19].

2.3. Bovine herpesvirus-1 (BHV-1) challenge and monitoring clinical disease

A licensed veterinarian, blinded to treatment groups supervised all treatments and was responsible for collecting clinical data. BHV-1 challenge with isolate 108 (5×10^6 plaque forming units (pfu)/animal) was performed as described previously [20]. The strain and dose of BHV-1 used for aerosol challenge was previously optimized to induce signs of clinical disease (fever and weight loss) in seronegative calves [21]. Body temperature was monitored with an electronic rectal thermometer and body weight recorded with an electronic scale. Rectal temperatures and body weight were measured daily, beginning the day of BHV-1 challenge. Nasal swabs for virus isolation and nasal tampons for the collection of nasal secretions were collected prior to BHV-1 challenge and for 12 days post-infection (pi) using methods described previously [20]. Animals with severe clinical disease were euthanized using humane end-points defined by the University of Saskatchewan Animal Protocol 19940218. Animals were euthanized by intravenous injection of 100 mg sodium pentobarbital/kg body weight (Euthanyl, Biomeda MTC, Cambridge, ON Canada) and submitted for autopsy and bacterial culture (Prairie Diagnostic Services Inc).

2.4. Enzyme-linked immunosorbent assays (ELISAs)

Nasal secretions for quantification of IgA and IFN production in the URT were collected as described previously [20]. Briefly, a single cotton tampon were inserted into the nostril for 20 min, absorbed fluid was expressed from the tampon, and transported to the lab on ice. Secretions were clarified by centrifugation at 2200*g* for 8 min at 4C and 200 μl aliquots, containing 1X protease inhibitor (P1860; Sigma-Aldrich, Burlington, MA), were stored at −80 °C. Different aliquots of each nasal secretion sample were used for IgA and IFN analysis to minimize protein degradation which may occur with repeat freezing and thawing of samples. ELISAs to detect IgA antibodies specific to the major BHV-1 envelope protein, glycoprotein D (gD), were performed as previously described [22] with modifications described by Hill et al. (2012) [15]. IFNαand -γ concentrations in nasal secretions were determined using a protein capture ELISA as previously described [23].

2.5. Quantifying BHV-1 in nasal secretions

Nasal secretions for analysis of virus shedding were collected from all animals the day prior to BHV-1 challenge and on day 2, 4, 6, 9, and 12 pi. Nasal secretions were collected with sterile cotton swabs and swabs were immersed in one ml Minimum Essential Medium (MEM) and transported on ice to the lab. Swabs were stored in MEM at −20 °C prior to performing viral plaque assays to quantify infectious BHV-1 particles. MDBK cells were used to perform plaque assays to quantify BHV-1 shed in nasal secretions as described previously [24].

2.6. Statistical analyses

Statistical analyses were performed using GraphPad Prism Version 6.00 software (GraphPad Software, Inc., San Diego, CA). A Shapiro-Wilk normality test was performed to determine whether parametric or non-parametric analyses were appropriate. Antibody titres and IFNα and -γ, were analyzed using a two-way ANOVA to determine if time and treatment had significant effects. When sig-

nificant treatment-dependent differences were identified then values for individual treatment groups were compared at each time point using a Dunn's Multiple Comparison Test. Body temperature and changes in body weight were analyzed with a Wilcoxin Signed rank test. Virus shedding data was log transformed to normalize data and then analyzed using two-way ANOVA to identify significant time and treatment effects. When significant treatment-dependent differences were observed then values for individual treatment groups were compared at each time point using a Dunn's Multiple Comparison Test. Significance was identified with $P < 0.05$.

3. Results

3.1. Presence of maternal antibody when vaccinating calves

Cows were parenterally vaccinated with MLV vaccine (Vista5 SQ) prior to breeding the previous year and passive transfer of neutralizing antibodies through colostrum was expected for all 5 viral components of the MLV vaccine. Neutralizing antibodies specific for all 5 viruses were detected in all 3–6 week old calves (Fig. 2A). SN titres varied among individual calves but there were no significant difference when comparing VN titres between the two treatment groups (data not shown) and data was then pooled for all animals in the study (Fig. 2A). Over 90% of 3 to 6 week old calves had BHV-1 VN titres higher than 1:10 and there were significant differences in VN titres when comparing among individual viruses. VN titres for BVDV1 (Mean = 1:3848; Range = 1:12 to 1;26,244) and BVDV2 (Mean = 1:2754; Range = 1:12 to 1;13,122) were similar but significantly ($P < 0.001$) higher than all other viruses. PI-3 titres (Mean = 1:1750; Range = 1:6 to 1;13,122) were also significantly ($P < 0.01$) higher than BHV-1(Mean = 1:230; Range = 1:4 to 1;1458) and BRSV . (Mean = 1:187; Range = 1:4 to 1;972). Differences in passively transferred VN titres, when comparing among the 5 viruses, are consistent with BHV-1 and BRSV being less immunogenic vaccine components than BVDV and these relative differences are consistent with results from a previous study [18].

Ellis et al. (2001) reported parenteral vaccination of neonatal calves with a MLV vaccine did not significantly alter decay of passively acquired maternal antibody specific for BVDV2 [12]. We also observed no significant difference in maternal antibody decay when serum samples were collected 4.5 months following IN delivery of either MLV vaccine or saline (data not shown). VN titres declined markedly for all viruses but VN titres for BVDV1 and BVDV2 remained significantly ($P < 0.01$) higher than the 3 other viruses (Fig. 2B). Over 85% (78/90) of 5 month old calves now had BHV-1 VN titres less than 1:10 (Fig. 2B). For the BHV-1 challenge component of the study (Fig. 1), we selected BHV-1 seronegative (Fig. 1B; VN titre less than 1:2) calves to ensure disease protection provided by immune memory was not confounded by the presence of VN maternal antibody. This selection criteria may result in an under-estimate of vaccine efficacy.

3.2. Clinical response to BHV-1 challenge

Aerosol challenge of naïve calves with virulent BHV-1 108 isolate results in fever and substantial weight loss within two to three days pi and this clinical disease persists for over 5 to 7 days [20]. In the current study, BHV-1 seronegative (VN titre <1:2) calves that were not vaccinated (Group A) displayed a significant ($P < 0.01$) increase in temperature on day 2 pi and temperatures remained significantly ($P < 0.01$) elevated throughout the pi period (Fig. 3A). A similar significant ($P < 0.01$) increase in temperature was observed for calves receiving a single IN vaccination with

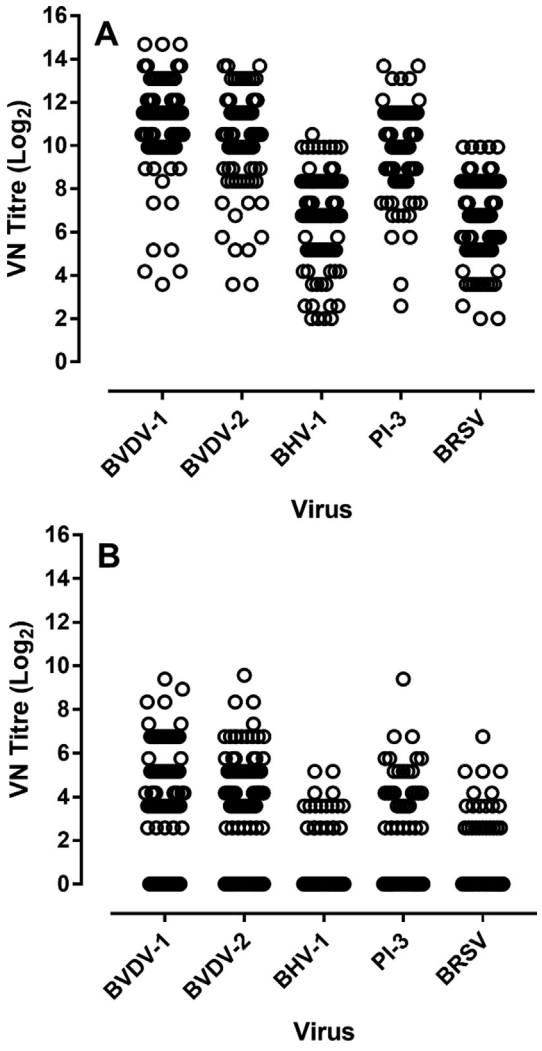

Fig. 2. Virus neutralization (VN) titres at time of primary intranasal vaccination and prior to vaccination and virus challenge at 6 months. Serum samples were collected from 3 to 6 weeks old calves (**A**) at the time of primary vaccination and then 4.5 months later (**B**), two weeks prior to vaccination and viral challenge. VN titres were determined for all viral components of the MLV vaccine, including bovine herpesvirus −1 (BHV-1), bovine viral diarrhea type 1 and 2 virus (BVDV-1 and BVDV-2), bovine respiratory syncytial virus (BRSV), and parainfluenza virus type 3 (PI-3). Student's t-test revealed no significant difference in VN titres when comparing the two treatment groups, intranasal saline (n = 45) and intranasal MLV vaccine (n = 45), at both times sampled. Data were pooled from both treatments and are presented as values for individual animals (n = 90).

the MLV vaccine either as neonates (Group B) or the day after weaning (Group D). A significant ($P < 0.05$) reduction in fever was observed for calves receiving IN MLV vaccination as neonates and a booster MLV vaccination, either IN (Group C) or SC (Group E). Thus, prior IN vaccination of neonatal calves was necessary for vaccination the day after weaning to effect a significant reduction in fever.

A different response pattern was observed when comparing weight loss among treatment groups following BHV-1 challenge. Weight loss for each calf was calculated by subtracting weight on each day pi from weight on the day of viral challenge. This eliminated individual animal variation in initial weight as a factor in the analysis. Naïve control calves (Group A) displayed significant ($P < 0.01$) decreases in weight on all days pi (Fig. 3B). When compared to Group A, calves receiving a single IN MLV vaccination as neonates (Group B) displayed a significant ($P < 0.05$) reduction in weight loss but calves receiving a single IN MLV vaccination the

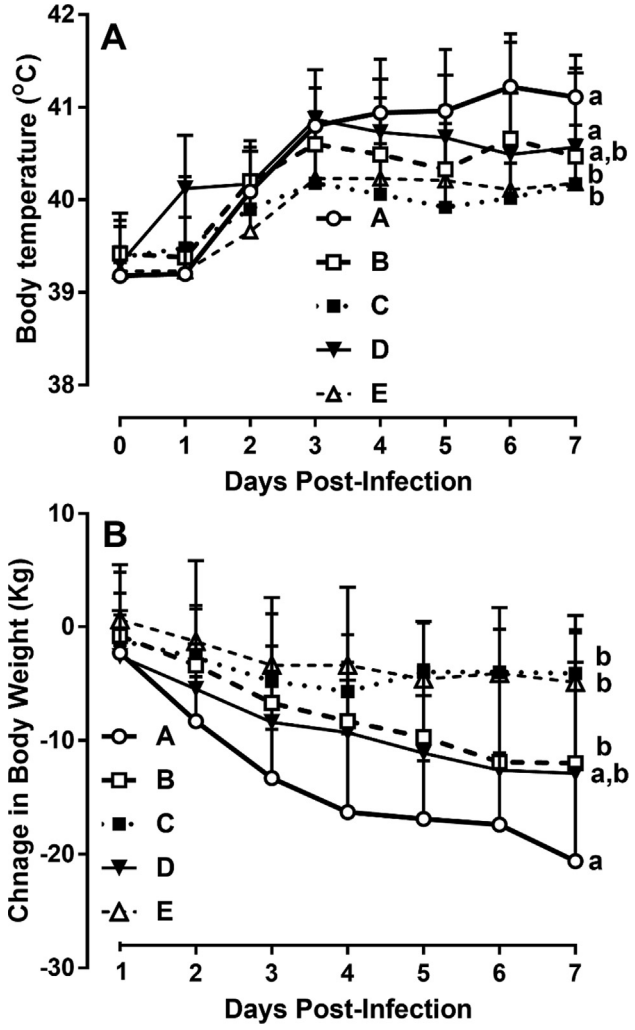

Fig. 3. Clinical response to BHV-1 aerosol challenge. Daily rectal temperature (**A**) are reported for the period during which all calves survived viral infection. Change in body weight (**B**) was calculated for individual calves relative to their body weight on the day of BHV-1 challenge. Experimental groups (n = 10/group) were: A) Naïve Controls; B) single IN MLV vaccination of neonatal calves; C) IN MLV vaccination of neonates and IN booster vaccination one day after weaning; D) primary IN MLV vaccination one day after weaning; and E) IN MLV vaccination of neonates and SC booster vaccination one day after weaning. Data presented are mean and 1SD. Treatment groups were compared using a two-way ANOVA for repeated measures with time and treatment as variables and significant ($P < 0.05$) differences among treatment groups over the study period are indicated by letters (a and b) at the end of each line.

day after weaning (Group D) did not display a significant ($P = 0.186$) reduction in weight loss. Calves receiving a booster vaccination, either IN (Group C) or SC (Group E) displayed highly significant ($P < 0.001$) reductions in weight loss. Thus, while a single IN MLV vaccination of neonates resulted in a significant ($P < 0.05$) reduction in weight loss when calves were infected 5 months later, the magnitude of this weight loss was further reduced with booster vaccinations 4 days prior to challenge (Groups C and E). Significant ($P < 0.01$) weight loss, relative to day 0, was observed on days 3 to 7 pi for Group B (single neonatal IN vaccination) but weight loss was not significant on any day pi for Group C (MLV + MLV) and significant only on day 4 pi for Group E (MLV + SC MLV). Thus, monitoring weight loss after BHV-1 infection provided evidence that immune memory persisted 5 months after a single IN vaccination (Group B). This immune memory also supported a rapid anamnestic response within 4 days after the booster vaccination that effectively reduced clinical disease, as measured by both fever and weight loss.

3.3. Virus shedding and secondary bacterial pneumonia

Aerosol challenge of naïve calves with virulent BHV-1 (108 isolate) causes infection throughout the URT [25] and infectious virus particles are shed in nasal secretions for a period of 10–12 days [20]. Naïve calves (Group A) had a significant ($P < 0.01$) increase in virus shedding on day 3 pi and continued to shed over one million virus particles/ml nasal secretion for the next 9 days (Fig. 4A). Relative to naïve Controls, there was no significant reduction in virus shedding in calves receiving either a single IN vaccination at 3–6 weeks (Group B) or a booster SC vaccination at 6 months (Group E). In contrast, IN booster (Group C) and primary IN (Group D) vaccination at 6 months significantly ($P < 0.01$) reduced virus shedding compared to groups A, B, and E. Further, the reduction in virus shedding was significantly ($P < 0.01$) greater in calves receiving an IN booster versus primary IN vaccination. Virus was also detected in nasal secretions of Groups C and D prior to BHV-1 challenge (Fig. 4A). This represents shedding of vaccine virus after IN vaccination and contributed to an increase in virus shedding relative to naïve Controls (Group A) during the first 3 days after BHV-1 108 challenge. A similar significant ($P < 0.05$) increase in virus shedding was also observed for Group E calves on day 1 pi. This may represent vaccine virus since parenterally injected

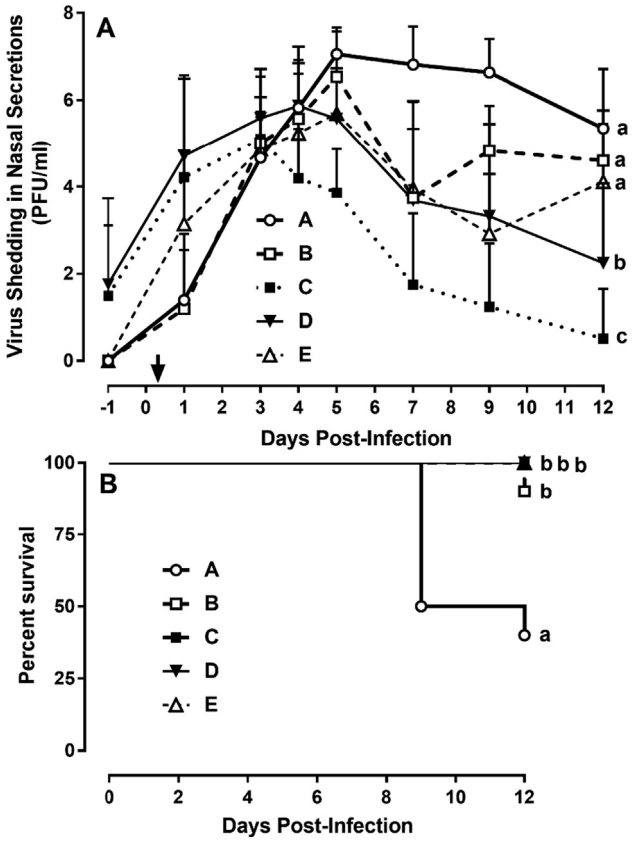

Fig. 4. Virus shedding in nasal secretions and calf survival following BHV-1 aerosol challenge. Shedding of infectious virus particles in nasal secretions (**A**) was quantified by plague-assay and percent survival (**B**) was calculated with a log-rank test. Experimental groups (n = 10/group) were: A) Naïve Controls; B) single IN MLV vaccination of neonatal calves; C) IN MLV vaccination of neonates and IN booster vaccination one day after weaning; D) primary IN MLV vaccination one day after weaning; and E) IN MLV vaccination of neonates and SC booster vaccination one day after weaning. Virus shedding data were log-transformed and presented as mean and 1SD. Treatment groups were compared using a two-way ANOVA with time and treatment as variables and significant ($P < 0.01$) differences among treatment groups over the study period are indicated by letters (a,b) at the end of each line.

modified-live BHV-1 can be translocated to and infect mucosal sites in the URT.

BHV-1 respiratory infections are an important factor contributing to secondary bacterial pneumonias [5], caused primarily by bacteria residing in the URT [26]. Following BHV-1 challenge of naïve Controls (Group A) there was 60% mortality (Fig. 4B) since calves were not treated with antibiotics. Post-mortem examination confirmed fibrinous pleuropneumonia was present and *Mannheimia (M.) haemolytica* was cultured from the lungs of all calves (data not shown). One calf in Group B and no calves in Groups C, D, and E developed fatal BRD following BHV-1 challenge. Log rank test analysis of calf survival revealed all four vaccine groups had significantly ($P < 0.001$) lower mortality when compared to naïve Controls and there were no significant differences among the vaccine groups.

3.4. Innate and acquired immune responses following BHV-1 infection

BHV-1 is a potent inducer of multiple types of interferon (IFN) following a primary infection in the URT [25]. On day 2 pi, IFNα (Fig. 5A) and IFNγ (Fig. 5B) were not significantly elevated in nasal secretions of naïve Controls (Group A) and vaccine groups, with the exception of Group D. Primary IN vaccination with the MLV vaccine 4 days prior to challenge (Group D) resulted in significantly ($P < 0.01$) elevated IFNα (Fig. 5A) and IFNγ(Figure 5B) levels on day 2 pi relative all other treatment groups. Further, calves receiving a primary IN vaccination at 6 months (Group D) maintained significantly ($P < 0.01$) higher IFNα (Fig. 5A) and IFNγ(Figure 5B) levels on day 5 pi than calves receiving booster vaccinations (Group C and E) but significantly ($P < 0.05$) lower IFNα and IFNγ level than naive Controls (Group A) and calves receiving a single IN vaccination at 3–6 weeks of age (Group B). Naive control calves (Group A) developed a further 5 to 10-fold increase in IFNα (Fig. 5A) and IFNγ(Figure 5B) production on day 5 pi when compared to day 2 pi. Significantly ($P < 0.05$) lower IFNα production but a similar increase in IFNγsecretion was observed in calves receiving a single IN MLV vaccination at 3–6 weeks of age (Group B) when compared to naïve Controls on day 5 pi. IFNα (Fig. 5A) and IFNγ (Fig. 5B) production did not increase significantly at any time pi in calves receiving either an IN or SC booster vaccination and IFN production in Groups C and E was significantly ($P < 0.01$) lower than naïve Controls (Group A) and vaccine groups B and D on day 5 pi. Thus, booster vaccination in groups C and E suppressed both the IFNα and IFNγresponses induced by BHV-1 infection.

Further evidence for an immune memory response following primary IN vaccination of 3–6 week calves was apparent when IgA antibody responses in the URT were analyzed (Fig. 5C). IgA antibody titres specific for tgD protein of BHV-1 were similar and very low in all treatment groups when calves were weaned at 6 months of age. Four days after vaccination at 6 months and the day calves were challenged with BHV-1 (Fig. 5C; Day 0) there was no evidence of an anamnestic IgA antibody response in any vaccinated group. There was, however, a significant ($P < 0.01$) increase in tgD-specific IgA antibody titres in nasal secretions of Group C (IN booster vaccination) two days after BHV-1 challenge (Day 2; Fig. 5C) and the magnitude of this antibody response increased further on day 5 pi. A significant ($P < 0.05$) increase in tgD-specific IgA antibody titre was also observed on day 5 pi when comparing over time within Group B (single IN MLV vaccination at 3 to 6 weeks). The increase in IgA titre on day 5 pi in Group B was, however, significantly lower than that observed for group C (Fig. 5C; Day 5). Thus, an IN booster vaccination (Group C) but not a SC booster vaccination (Group E) induced rapid onset of increased IgA production in the URT.

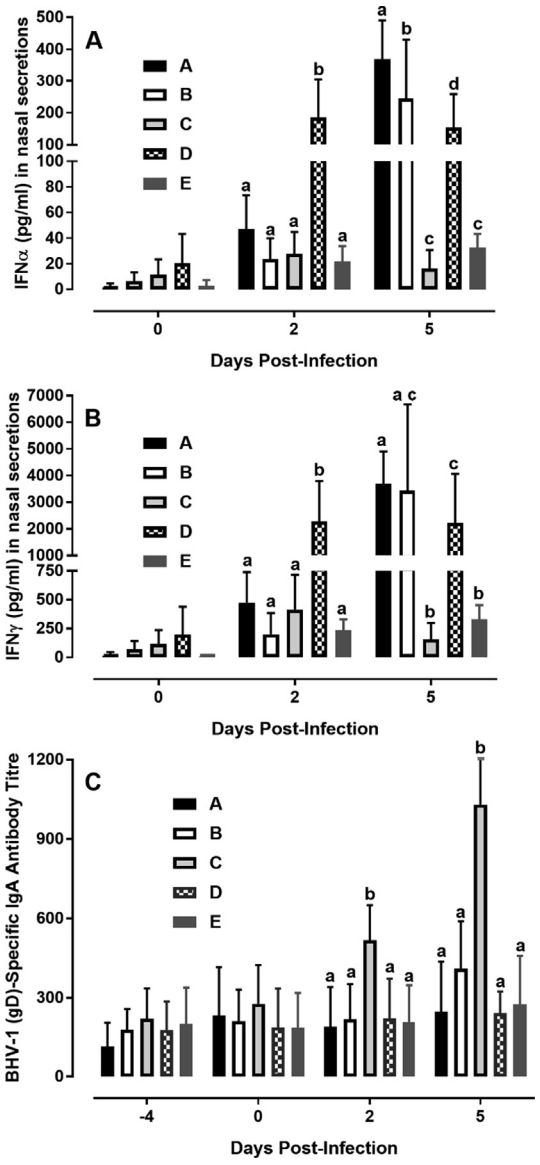

Fig. 5. Innate and acquired immune responses following BHV-1 challenge. IFNα (**A**), IFNγ (B), and IgA antibody titres specific for the tgD protein of BHV-1 (**C**) were quantified in nasal secretions by ELISA. Experimental groups (n = 10/group) were: A) Naïve Controls; B) single IN MLV vaccination of neonatal calves; C) IN MLV vaccination of neonates and IN booster vaccination one day after weaning; D) primary IN MLV vaccination one day after weaning; and E) IN MLV vaccination of neonates and SC booster vaccination one day after weaning. Treatment groups were compared using a two-way ANOVA with time and treatment as variables and significant ($P < 0.01$) differences among treatment groups at each time point are indicated by letters (a–d).

4. Discussion

Immune memory following vaccination is a key factor influencing both duration of disease protection and the requirement for booster vaccinations to maintain a protective level of immunity. Protective immunity due to IgA secretion at mucosal surfaces may wane relatively quickly following IN vaccination with a MLV vaccine [14] but long term persistence of memory B and T cells has been reported following mucosal vaccination [27,28]. The current study provides evidence that a single IN injection of a MLV vaccine in neonatal calves induced mucosal immune memory to BHV-1 that persisted for at least 5 months. This mucosal immune memory supported rapid onset of an anamnestic response after

an IN booster vaccination that was characterized by enhanced IgA secretion (Fig. 5B) and a significant ($P < 0.01$) reduction in both clinical disease (Fig. 3) and shedding of infectious virus (Fig. 4). Induction of an anamnestic response was confirmed through comparison with a primary IN MLV vaccine also given four days prior to BHV-1 challenge. The primary IN vaccination at 6 months did not significantly reduce clinical disease and was associated with a significantly ($P < 0.05$) lower reduction in virus shedding when compared to the IN booster vaccination. The single IN vaccination at 6 months was able, however, to significantly reduce mortality due to a secondary bacterial pneumonia. Thus, primary IN vaccination of neonatal calves, despite the presence of VN maternal antibody, provided sufficient immune memory in 5 to 6 month old calves to prevent clinical disease, viral infection, and secondary bacterial pneumonia within four days after an IN booster vaccination.

Head restraint and accurate delivery of IN vaccines can be more difficult in 5 to 6 month old calves. Therefore, we compared the capacity of both IN and SC booster vaccinations to induce protective immune responses at 6 months of age. SC and IN booster vaccinations were equally effective when comparing the reduction in clinical disease (Fig. 3) and secondary bacterial pneumonias (Fig. 4B). The SC booster vaccination, however, failed to induce a significant reduction in virus shedding (Fig. 4A) or enhance IgA production in the URT (Fig. 5B). Secretory (S)IgA is an important effector mechanism involved in virus neutralization at mucosal surfaces and the failure of SC vaccination to increase BHV-1-specific SIgA in nasal secretions is consistent with a failure to significantly reduce virus shedding. The observation that a SC booster vaccination significantly ($P < 0.01$) reduced clinical disease (Fig. 3) but not viral shedding raises the possibility that apparently healthy calves may be a source of infection for other animals. Thus, while a vaccination protocol may provide an effective aid in the prevention or reduction of clinical disease further studies may be warranted to confirm vaccination also prevents disease transmission. A reduction in clinical disease without a reduction in the duration of infection and pathogen shedding was reported for a human vaccine targeting a bacterial respiratory pathogen [29]. This situation may have important implications for disease transmission and maintenance of a pathogen within an apparently healthy population [30].

The reduction in clinical disease, without a reduction in viral infection, observed following the SC booster vaccination may be consistent with the significant ($P < 0.01$) reduction in IFN production observed for this group (Fig. 5A and B). Studies with recombinant bovine IFNs confirmed type I and II IFNs induce fever and anorexia with weight loss in cattle [21,31]. Primary BHV-1 infection induces increased production of multiple IFN types in the URT [25] and increased IFN production was observed in naïve calves following challenge with virulent BHV-1 (Fig. 5A and B; Group A). A primary IN MLV vaccination of naïve calves at 6 months also induced an accelerated onset of increased IFN production on day 2 pi, which is characteristic of another commercial modified-live BHV-1 vaccine [32]. Increased IFN production may be one reason the primary IN MLV vaccination was not associated with a significant reduction in clinical disease. Immune memory from the primary IN MLV vaccination of neonatal calves was sufficient to significantly reduce IFNα but not IFNγ production following BHV-1 challenge (Fig. 5A and B; Group B) and this group was associated with some reduction in weight loss but no reduction in fever. Thus, only IN and SC booster vaccinations were able to significantly inhibit both IFNα and IFNγ responses to viral infection and significantly reduce both fever and weight loss Modulation of innate immunity by acquired immunity is an unusual observation but there is increasing evidence that acquired immunity, through the activity of regulatory T cells (Tregs), may play an important role in controlling a wide variety of innate immune functions [33]. The current study clearly suggests acquired immunity can also inhibit IFN production, a key innate mucosal immune response during viral infection. Inhibition of IFN responses could not be explained by a simple reduction in viral infection since IFN secretion was suppressed following SC vaccination, which did not significantly reduce virus shedding. Further studies are required to determine whether non-conventional T cells, the major cell type recruited to the URT during BHV-1 infection [34] and a known source of IFNγ [35], are regulated by vaccine induced Tregs.

Primary BHV-1 infection is a critical component in development of fatal secondary bacterial pneumonias caused by *M. haemolytica* [20]. The current study did not involve a secondary bacterial challenge but 60% of naïve calves developed fatal bacterial pneumonia within 9–12 days after BHV-1 infection (Fig. 4B; Group A). Fatal secondary bacterial pneumonia, caused by *M. haemolytica*, is consistent with current information that this bacteria is a common resident in the URT of young calves [26] and causes secondary bacterial pneumonia when pulmonary defenses are compromised [36]. It is important to note that secondary bacterial pneumonias were significantly ($P < 0.01$) reduced in all treatment groups that received a primary IN MLV vaccine, either at 3–6 weeks of age or at 6 months of age (Fig. 4B). Multiple mechanisms have been proposed by which BHV-1 infection may enhance bacterial colonization of the lung or alter host responses following bacterial infection [3,20]. Memory B cells may persist for a prolonged period in the lung [27] and sufficient immune memory may have persisted following primary IN vaccination of neonatal calves to prevent BHV-1 infection in the lungs but not the URT. Further studies are required to determine whether memory B cells, either IgG or IgA, were induced by IN vaccination of neonatal calves and if these pulmonary memory B cells persisted at a level sufficient to prevent or reduce BHV-1 infection.

Vaccine efficacy observed in the present study reflects protection from clinical disease and BHV-1 infection following aerosol challenge with a high dose (5×10^6 pfu) of a virulent BHV-1 field isolate [21]. This challenge results in BHV-1 infection throughout the URT, trachea and lungs of calves [34]. Different clinical disease and virus infection outcomes may occur if calves are challenged with either lower doses or different BHV-1 isolates. Fairbanks et al. reported significant reductions in clinical disease and BHV-1 shedding following a primary parenteral injection of a multivalent MLV vaccine 3–4 days prior to BHV-1 aerosol challenge [37]. Calves in this study were challenged with 10-fold less virus than used in the current study, the Cooper lab strain of BHV-1 was used for the challenge, and calves were treated with antibiotic to prevent secondary bacterial infections [37]. The current study may, therefore, underestimate disease protection provided by both the primary IN vaccinations and IN and SC booster vaccinations if weaned calves are treated with antibiotics and exposed to a low infectious dose of BHV-1. Further, the MLV vaccine used for IN vaccination in the current study does not represent currently licensed bovine IN vaccines, which do not contain BVDV1 and 2. The presence of BVDV in the MLV vaccine is not expected to alter the efficacy of the BHV-1 component, however, since a previous study confirmed this vaccine strain of BHV-1 induced protective immunity in young calves when BVDV1 and 2 were vaccine components [16].

In conclusion, the present study provides evidence that a single IN vaccination of neonatal calves, despite the presence of neutralizing maternal antibody, induced BHV-1 specific mucosal immune memory with a 5 month duration. This immune memory effectively blocked the viral-bacterial synergy that is an important component of BRD but an IN booster vaccination was required to ensure rapid onset of protective immunity in the URT that was able to prevent both clinical disease and significantly reduce virus

shedding following a respiratory BHV-1 challenge. A SC booster vaccination also prevented clinical disease but did not significantly reduce the level of virus shedding from the URT. These observations provide important information that can be used to design strategic vaccination programs to further improve BRD control in the post-weaning period. Under current management programs, calves are often accessible for IN vaccination early in life and when weaned at 5 to 6 months of age. A primary IN vaccination early in life and an IN booster vaccination at weaning is compatible with prior vaccination of the dam and ensures rapid onset of protective immunity during the post-weaning period when cattle are at greatest risk for viral infections which cause BRD.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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