RESPIRATORY VACCINATION STRATEGIES TO ENHANCE MUCOSAL AND SYSTEMIC IMMUNE RESPONSES IN BEEF CALVES

by

Heather Deann Hughes

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Approved as to style and contents by:

_____	_____
Dr. John T. Richeson Chairman, Dissertation Committee	Date
_____	_____
Dr. Jeff Carroll Member, Dissertation Committee	Date
_____	_____
Dr. Pablo Pinedo Member, Dissertation Committee	Date
_____	_____
Dr. Mallory Vestal Member, Dissertation Committee	Date
_____	_____
Dr. David Parker Member, Dissertation Committee	Date

_____	_____
Head, Major Department	Date
_____	_____
Dean, Graduate School	Date

ABSTRACT

Prevention of bovine respiratory disease (BRD) in the U. S. beef industry is typically approached using vaccination strategies upon arrival to the feedlot, since vaccination at the cow-calf sector has a low adoption rate. Four experiments were conducted to explore novel vaccination routes and viral-bacterial combination vaccines in cattle. Experiments 1 and 2 evaluated novel mucosal vaccination routes and experiments 3 and 4 compared two combination viral-bacterial respiratory vaccines. In experiment 1, 42 crossbred beef steers were administered 1 of 5 treatments, consisting of three titrated oral doses of pentavalent modified live viral respiratory vaccine, a label administration of injectible pentavalent vaccine, or saline administered orally as a negative control treatment. Results indicated no immunological benefit to oral vaccination of calves with a pentavalent respiratory vaccine administered orally. In experiment 2, 30 healthy beef heifer calves were administered a commercially available intranasal trivalent modified live respiratory vaccine, a pentavalent respiratory vaccine labeled for parenteral administration but administered intranasally, or saline administered intranasally. Serum and mucosal antibodies were undetectable in cattle treated with intranasal administration of a vaccine labeled for parenteral vaccine, which could be caused by interference of the adjuvant in the vaccine. Experiments 3 and 4 observed the effects of two different combination viral-bacterial respiratory vaccines containing *Mannheimia haemolytica* components compared to an unvaccinated negative control group. In experiment 3, cattle on either combination vaccine treatment elicited greater leukotoxin antibody responses on

d 7, 14, and 28 post-vaccination compared to control calves, suggesting that the two vaccines are comparable in protection against leukotoxin produced by *Mannheimia haemolytica* bacteria. The acute phase response and performace were evaluated following the same comparison in experiment 4. Cattle receiving Pyramid 5 + Presponse SQ had greater concentrations of haptoglobin compared to control cattle, with Bovi-Shield Gold 5 One Shot treatment being intermediate, which could suggest a greater inflammatory response stimulated by the Presponse vaccine. No differences in performance were observed. Further research investigating these vaccination strategies could lead to the implementation of products at the cow-calf sector that reduce BRD prevalence and minimize morbidity and mortality in the feedlot attributable to BRD.

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DEDICATION

I dedicate this work to my children, Mia and Hudson Hughes. You are the lights of my life; my pride and joy. May you always know how dearly I love you and support you in your endeavors, and above all, may you seek the Lord first in all of life's pursuits.

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CHAPTER I

REVIEW OF LITERATURE

Bovine Respiratory Disease

Beef Production System of the United States

Bovine respiratory disease (BRD) is the most common and costly disease within the North American beef industry (Smith, 1998; NAHMS, 2000a), and the structure of the beef production system largely contributes to this long-lasting phenomenon. Beef production in the U.S. is highly segmented, originating at the cow-calf operation, proceeding to either a stocker operation or feedlot, and transitioning finally to the packer. Small cow-calf operations, which have less than 100 cows, account for approximately 90.4% of all farms with beef cows and supply 45.9% of beef cows in the United States (USDA - NASS). Commonly, cattle on these operations are channeled through conventional beef markets, rather than forward priced, and a large percentage of these calves have never received a vaccination against respiratory or other pathogens (USDA - APHIS, 2011).

In 2007, only 26 % of cow-calf operations with less than 50 cows, and 63 % of operations with between 50 and 99 cows, vaccinated cattle or calves against respiratory disease before sale (USDA-APHIS, 2011). Additionally, the majority of small cow-calf farms are located in the southeastern U.S.; therefore, a large percentage of calves entering the feedlot are not only unvaccinated but are also often transported a long distance to feedlots that are concentrated in the High Plains region of the U.S. Aside from purchase

origin, most feedlot personnel have no knowledge of cattle health or management history, with only 32.4% of feedlots receiving this information (Duff and Galyean, 2007). One method for the feedlot sector to ensure that newly received cattle have been vaccinated and managed appropriately is to purchase preconditioned calves.

Preconditioning

Generally, preconditioned cattle are weaned for a minimum amount of time (usually 30 to 45 d), vaccinated with clostridial and respiratory vaccines, treated with anthelmintic, castrated (if applicable), dehorned, and acclimated to feed bunks and water troughs (Duff and Galyean, 2007). This process adds value to the animal by improving its health and performance. Significant differences in performance of preconditioned calves have been reported by feedlot producers which include lower death loss, reduction in illness, greater ADG, improved feed efficiency, and superior carcass traits (Avent et al., 2004).

Richeson et al. (2013) observed greater total morbidity in newly weaned beef steers and bulls sourced from auction markets when compared to preconditioned cattle, with a morbidity rate of 70.4 % in auction market animals versus 6.7 % in preconditioned. Additionally, preconditioned calves gained 1.2 kg/d compared to 0.85kg/d for auction market calves. Based on a survey of feedlot producers, most believe that preconditioning cattle is beneficial in decreasing morbidity and mortality in calves weighing less than 318 kg (USDA – APHIS, 2000a). Despite this common assertion, many stockers and feedlot producers do not purchase preconditioned calves because of higher purchase cost, and in addition, many small-cow producers do not precondition calves; therefore, the market availability of appropriately managed cattle is limited. For

the calf supplier, preconditioning requires additional labor, adequate facilities, and additional costs and risks associated with time of ownership, making a greater net return essential for adoption of the practice.

Economics of Preconditioning

Price differentials for preconditioned feeder cattle are reported in several earlier studies (Buccola, 1980; Marsh, 1985; Faminow and Gum, 1986; Lambert et al., 1989; Turner et al., 1991; Schroeder et al., 1998; Smith et al., 2000). Attributes of these cattle include weaned for 45 d or longer, castrated bulls, polled or dehorned animals, improved condition or fleshiness, healthy appearance, and often larger, more uniform lots of cattle. Some of these items may caution the cow-calf producer. For example, cattle that weigh more typically bring lower prices per unit of body weight, and cattle which appear fleshy may be unappealing to the stocker or feedlot operator who perceives fleshy cattle as lacking opportunity for compensatory weight gain (Wren, 2011).

Ward et al. (2007) developed two models, using price premium data from 20 auction sales in eight livestock market locations over a two-year period. Their research addresses the price premium risk for marketing preconditioned calves and considers the benefits from preconditioning and marketing cattle in larger sale lots. Avent et al. (2004) estimated premiums across calf sale data from three consecutive d and observed a $3.36/cwt premium over regular weekly auction prices for cattle originating from single-protocol sources (i.e., the calf supplier followed a single, set protocol). From the same data set, cattle on multiple-protocol programs, in which vaccination and weaning guidelines varied, were marketed at an estimated $1.96/cwt premium over regular auction

prices. The differences in these premiums are likely due to more stringent guidelines for the single-protocol programs.

Feeder calf characteristics from two sales per year over a five-year period were analyzed by Dhuyvetter et al. (2005), with sales taking place in either fall or winter seasons. On average, preconditioned calves received a $4.62/cwt premium in the fall sales and a $3.22/cwt premium in winter sales. Variability was present across years and time of year, with fall premiums over the entire time period ranging from $3.90 to $5.45/cwt and winter premiums ranging from $2.30 to $4.63/cwt. King and Seeger (2004) developed models to estimate price premiums from feeder cattle data derived from ten sales between 2003 and 2004. Two preconditioning programs generated premiums of $5.33 and $4.84/cwt.

Superior Livestock Auction's video sales from each year between 1995 and 2004 were used to estimate premiums paid for preconditioned cattle from three value-added programs (King and Seeger, 2005). The most stringent preconditioning program produced the highest premium, with added values of this program ranging from $2.47 to $7.91/cwt; an average premium of $4.37/cwt was estimated over the entire ten year observation period. In terms of stringency and management requirements, the intermediate program averaged $1.91/cwt for the ten years, and the least stringent program, with the fewest management requirements, had an average premium of $1.07/cwt for the ten-year period.

Based on these data, buyers consistently pay premiums for preconditioned cattle; however, those premiums are quite variable. Time of year, location, and stringency of the value-added programs all have an impact on premium. In the study conducted by

Ward et al. (2007), data from twenty individual sales from eight locations were analyzed using two methods, and the variation in premiums evident in the prior research was again present. This raises questions with regard to producer (seller) risk associated with investing in preconditioning. Factors in this study which impacted premium price were numerous and included: lot size, with high premiums paid for sale lots larger than ten animals; weight, with premiums decreasing with increasing lot weight; breed, with buyers paying more for continental breeds; sex, with heifers having lower premiums across all sales; condition, with thin cattle receiving smaller premiums and fleshy cattle receiving discounts; frame size, with larger framed cattle receiving discounts; uniformity, with uneven lots receiving discounts; health, with unhealthy cattle receiving discounts; and horns, with cattle receiving discounts for not being dehorned (Ward et al., 2007).

During the three-year period, premiums ranged from $1.87 to $13.73/cwt. Across years, the average premiums were $1.04, $4.85, and $4.38/cwt for year one, two, and three, respectively. Impacts not accounted for in this analysis are buyer competition, which varies from sale to sale, and reputation of the cattle sources, which likely plays a role in determining premiums (Turner et al., 1993). These data illustrate that production risk accompanies price premium risk for the cow-calf operator. Net returns vary widely among these data, from a loss of $7.49/hd to a gain of $43.48/hd. According to the budget designed by Ward et al. (2007), a breakeven premium of $1.36/cwt exists, which should encourage adoption, since it is well below the average premiums, yet profitability from value added practices is not guaranteed.

The current body of literature on the economic net return of preconditioning programs is small. Evidence does suggest that economic justification varies based on

program, size of the lot, characteristics of the cattle, time of year, and even reputation of the seller. For many, it pays to precondition, yet business decisions and capital flow ultimately drive the markets.

At the current time, for the cattle producer who owns a small number of cows and holds a "nine-to-five" job, it is likely not economically feasible or profitable to invest the time and capital to meet the requirements that generate higher premiums. Many small producers do not have the facilities, labor or knowledge required to vaccinate calves. Further, purchasing feed, feed bunks, vaccines, etc. are an added cost that offsets the potential premiums offered for preconditioned calves. Additionally, the added risk of death-loss during the 30-45 d weaning program in a preconditioning protocol is not accounted for in these economic analyses. These hurdles have likely perpetuated the persistence of BRD prevalence in the feedlot sector and have created a need for vaccination strategies that are simple to implement at the cow-calf sector.

Pathogenesis of BRD

The contribution of the segmented beef production system to BRD prevalence in the feedlot is perhaps better appreciated when accompanied by an understanding of the pathogenesis of this complex disease. Typically, in severe cases of the disease, three primary factors contribute: physiological stress, viral pathogens, and bacterial pathogens (Bagley, 1997).

Viral Pathogens

The primary viruses implicated in BRD complex include bovine herpesvirus-1 (BHV-1; Jericho and Langford, 1978), bovine respiratory syncytial virus (BRSV; Durham et al., 1991; Gagea et al., 2006; Hagglund et al., 2007), parainfluenza-3 virus

(PI-3V; Fulton et al., 2000;Cusack et al., 2003; Juarez et al., 2003), and bovine viral diarrhea virus genotypes I and II (BVDV I and II; Haines et al., 2001; Shahriar et al., 2002; Reggiardo et al., 2005; Gagea et al., 2006; Booker et al., 2008; Booker et al., 2008). At times, more than one of these viruses, in concert, are isolated from animals afflicted with BRD (Martin and Bohac, 1986; Richer et al., 1988). While these five viruses are most commonly identified from sera, nasal isolates, or lung tissue of affected animals, recent investigations suggest that bovine coronavirus may also be implicated in BRD complex (Martin et al., 1998; Lathrop et al., 2000; Plummer et al., 2004; Storz et al., 2007; Decaro et al., 2008).

Respiratory viruses may cause clinical BRD in an uncomplicated manner, or contribute to BRD pathogenesis indirectly by predisposing the animal to bacterial lung infection (i.e., bronchopneumonia) in two distinct ways. First, viral pathogens may directly damage the respiratory clearance mechanisms and lung parenchyma, which allows for commensal bacteria in the upper respiratory tract to migrate ventrally into the lower respiratory tract and establish infection in the compromised lung (Martin and Bohac, 1986). Secondly, when a primary viral infection is present, the immune system's ability to respond to bacterial growth or infection is compromised; therefore, pathogenic bacteria can more easily proliferate and infect the lung (Czuprynski et al., 2004). Furthermore, BVDV has the unique ability to cause persistent infection of calves *in utero* when the dam is exposed (Taylor et al., 2010). These calves are referred to as persistently infected or "PI" calves and are a primary source of BVDV transmission in the feedlot (Fulton et al., 2005).

Multiple studies have demonstrated moderate to high prevalence (27 to 64% of cases) of BVDV in BRD mortalities (Haines et al., 2001; Haines et al., 2004; Shahriar et al., 2002). With regard to performance, Burciaga-Robles et al. (2010) reported tendencies for decreased ADG and G:F across the finishing period in steers exposed to PI cattle. Richeson et al. (2013) observed immunomodulation in cattle exposed to PI calves, with exposed cattle tending to express greater serum TNF-α concentrations and greater IFN-γ and IL-6 concentrations. Exposure to PI cattle also impacted performance following feedlot arrival, with reduced BW gain from d 28 to 42, and health, with a greater number of PI-exposed calves treated thrice for BRD compared to control animals. It is important to note that in this study, cattle management strategies served as factors (i.e., either preconditioned or auction market-derived), and PI calf exposure did not clearly elicit immunomodulation in preconditioned cattle.

The five aforementioned viruses often play a significant role in the initiation of bacterial infection associated with BRD, and are the typical agents included in commercial modified-live virus vaccines administered on-arrival in the feedlot.

Bacterial pathogens

The primary bacterial pathogens associated with BRD are *Mannheimia haemolytica* (Jubb and Kennedy, 1970; Mosier et al., 1989; Wilson, 1989) and *Pasteurella multocida* (Lillie, 1974; Watts et al., 1994; Fulton et al., 2000; Welsh et al., 2004). *Mannheimia haemolytica* exists as commensal bacteria of the upper respiratory tract and nasopharynx of healthy, immunocompetent bovine animals (Frank, 1989; Carter et al., 1995); however, it is also the major bacterial agent of BRD complex, making it a pathogen of considerable economic importance in the North American feedlot industry.

This bacterium is a Gram-negative, non-motile, non-spore-forming, fermentative, oxidase-positive, facultative anaerobe (Quinn, 1994; Hirsh and Zee, 1999) and resides primarily in the nasopharynx (Babiuk and Acres, 1984) and tonsils (Frank et al., 1995) in healthy calves. Under homeostatic conditions, it maintains a symbiotic relationship with its host; yet, viral infection, as described previously, or physiological stress, which will be covered in a subsequent section, may cause this otherwise benign organism to become pathogenic (Farley, 1932; Blood et al., 1983).

The frequency of *M. haemolytica* presence in the naris increases as cattle move into the feedlot segment of beef production (Frank et al., 1994), which poses the question: Which events leading up to feedlot entry contribute to the transition of bacteria from commensal to pathogenic? While a number of virulence factors have been proposed, ruminant-specific leukotoxin (Lkt) is believed to be central in the pathogenesis of *M. haemolytica*. Leukotoxin effects range from the impairment of function to lysis of ruminant leukocytes (Baluyut et al., 1981; Berggren et al., 1981; Shewen and Wilkie, 1982; Gentry et al., 1985; Clinkenbeard et al., 1989; DeBey et al., 1996). Once the bacterium moves deeper into the lower respiratory tract, Lkt plays a major role in inducing lung injury and allowing bacteria to thrive by evading phagocytotic cell destruction (Tatum et al., 1998). Bovine leukocytes are particularly sensitive to Lkt (Clinkenbeard et al., 1989; Clinkenbeard et al., 1989; Iwase et al., 1992; Maheswaran et al., 1992; Marshall et al., 2000). Additionally, Czuprynski et al. (2004) reported that viral infection increases this susceptibility of bovine leukocytes to Lkt. Studies on target cells have demonstrated that Lkt is highly toxic to ruminant polymorphonuclear (PMNs) cells, moderately toxic to horse PMNs, and nontoxic to swine and rabbit PMNs (Tan et

al., 1994); however, the mechanism by which Lkt is particularly lethal in ruminant leukocytes has not been elucidated (Narayanan et al., 2002). The role of Lkt will be further explored, in Chapters 3 and 4, as it is a component in the combination respiratory vaccines evaluated in two of the studies comprising this dissertation.

Pasteurella multocida is also a primary bacterial pathogen associated with BRD complex and has been isolated in cattle received at the feedlot in numerous studies, via isolation from the lungs of fatal cases (Watts et al., 1994; Fulton et al., 2000; Storz et al., 2000a; Welsh et al., 2004; Gagea et al., 2006). Extensive explanation of *P. multocida* is beyond the scope of this dissertation; however, though *M. haemolytica* is more commonly isolated in BRD-diagnosed feedlot cattle, mortalities associated with *P. multocida* appear to be increasing (Welsh et al., 2004).

Physiological stress

As previously mentioned, viral infection is often a pre-disposing factor in fatal bacterial respiratory infections. This phenomenon is commonly referred to as viral-bacterial synergy and is further enhanced by stress, which plays a significant role in incidence and severity of respiratory infections in cattle (Hodgson et al., 2005).

The first connections between stress and respiratory infections were elucidated in human studies, where a variety of stressors led to increased incidence and severity of respiratory infections (Boyce et al., 1977; Graham et al., 1986; Clover et al., 1989). Studies in mice have also revealed complex interactions between stressors and respiratory infections. The T helper 1- and T helper 2-type cytokine concentrations were reduced in mice that were restrained prior to viral challenge with influenza A. Additionally, restrained mice experienced reduced cellular infiltration in lungs and draining lymph

nodes (Hermann et al., 1993; Grutz, 2005). This experimental model suggests that an induction of elevated glucocorticoids (i.e., corticosterone), stimulated by restraint stress, led to a reduction in lung lesions and decreased mortality following respiratory infection (Dobbs et al., 1996). Conversely, in a study where the stressor was social reorganization prior to influenza A challenge, an increase in cellular infiltration in the lung and mortality were observed, possibly due to glucocorticoid insensitivity (Sheridan et al., 2000). These murine studies demonstrate how different psychological responses can induce differential effects on hosts with concurrent respiratory infection.

Likewise, cattle are exposed to a variety of stressors during the transition from the cow-calf operation to the feedlot. These include abrupt weaning, the transportation process, castration, temperature fluctuations, inadequate nutrition, and commingling/social restructure (Carroll, 2014). All "stress" is not equal and can be classified as either acute or chronic. Chronic stress (typically $\geq$ 12 hours) elicits immunosuppression, whereas acute stress (< 12 hours) can actually prime the immune system (Dhabhar, 2009). Understanding of the type of stress condition becomes important in the feedlot where immunological function is of utmost importance because immunocompromised cattle tend to have decreased growth performance and feed efficiency (Crookshank et al., 1979; Rulofson et al., 1988; Lay et al., 1992; Carrasco and Van De Kar, 2003; Charmandari et al., 2005; Buckham et al., 2008).

According to Chrousos and Kino (2005), the most current definition of stress is a "state in which homeostasis is threatened or perceived to be so; homeostasis is re-established by a complex repertoire of behavioral and physiological adaptive responses of the organism." With regard to BRD, the impacts of chronic stress have been observed to

alter the immune system, subsequently enhancing viral-bacterial synergy (Hodgson et al., 2005). Initially, the stress response is induced by neurons in the brain which ignite the release of corticotropin-releasing hormone (CRH) and vasopressin (VP) in response to stressful stimuli, such as those previously mentioned. Subsequently, adrenocorticotropin hormone (ACTH) is released from the anterior pituitary (Aguilera, 1998; Carrasco and Van De Kar, 2003), leading to the release of glucocorticoids (e.g., cortisol, in cattle) from the adrenal cortex (Markava et al., 1981; Carrasco and Van De Kar, 2003). This progression of the release of cortisol is referred to as "activation of the hypothalamic-pituitary-adrenal (HPA) axis" (Carroll, 2014). If this release of glucocorticoids is chronic in nature, as is often the situation during the relocation process over multiple d or weeks of transition for a calf, the immune system will be suppressed (Salak-Johnson and McGlone, 2007).

Stress/immune interactions

Suppression of the immune system following increased synthesis and release of stress-related hormones has been demonstrated in numerous trials. *In vitro* incubation of cattle and porcine immune cells with cortisol has demonstrated suppression of lymphocyte proliferation, IL-2 production, and neutrophil function (Wesley and Kelley, 1984; Blecha and Baker, 1986; Salak et al., 1993). Similarly, Blecha and Baker (1986) observed reduced lymphocyte proliferation, IL-2 production, and antibody production in cattle injected with ACTH to stimulate glucocorticoid synthesis and release. These suppressive effects of stress hormones on the innate and adaptive immune responses certainly impact the animal's ability to mount the appropriate immune response necessary to battle a viral infection, thus making the animal more susceptible to bacterial infection

in the lung. To demonstrate this, Babiuk et al. (1987) developed a disease challenge model in which a primary BHV-1 infection was followed by aerosol challenge with *M. haemolytica.* Further, abrupt weaning followed by commingling (defined as "social reorganization") and transportation were compared to transportation alone, immediately prior to dual BHV-1 and *M. haemolytica* challenge and the severity of secondary infection was monitored via clinical signs and mortality. In calves experiencing both social reorganization and transportation, mortality due to BRD was increased two-fold compared to cattle experiencing transport stress alone (80 vs. 40%). Stress and immune interactions are very complex, and while each animal entering the feedlot does not experience the same stressors, chronic stress impairs an animal's ability to withstand disease via immunosuppression.

Common stressors in the beef industry

As previously stated, multiple factors contribute to stress in the bovine, and these may be classified as environmental- or management-associated. Environmental effectors include endemic pathogen loads, transportation and weather, while castration, commingling different sources of animals, dehorning, and calf origin are management factors.

Transportation

Much research has been conducted to observe the impacts of transportation, or shipping, on cattle, since virtually all feedlot cattle have been transported at least once in their lifetime (Taylor et al., 2010). Various studies have resulted in conflicting results when investigating the association of distance traveled, or time in transit, with BRD. One large survey reported a positive correlation between distance traveled and clinical

morbidity (Sanderson et al., 2008). Similarly, Pinchak et al. (2004) associated disease incidence with distance traveled; however, confounding factors such as age of animal and castration status were not considered. Conversely, Ribble et al. (1995) observed no association between distance traveled and BRD incidence. Controlled studies, in which transport time is considered treatment, have produced similar results. Cole et al. (1988) reported that calves transported 12 h compared to those transported 24 h had higher morbidity levels and concluded that the sorting and loading aspects of transportation are most stressful. Other studies support this conclusion that handling may be more impactful than transportation itself (Stermer et al., 1982; Warris et al., 1995).

Weather

Traditionally, it has been accepted that fall is the season of greatest BRD incidence, implicating weather as a predisposing factor. However, this is confounded by the fact that fall is the season for marketing beef cattle in North America, resulting in more calves being commingled through marketing channels (Ribble et al., 1995; Loneragan et al., 2001). Rather than extreme weather conditions, such as persistent heat or cold, many authors have pointed to sudden and extreme changes in weather as being more predisposing to disease (Mac Vean et al., 1986; Alexander et al., 1989; Ribble et al., 1995).

Castration and dehorning

Management tools in the feedlot include castration and dehorning practices to reduce aggressive behavior and bruising/injury, respectively. Association between castration and BRD has not been consistent, and often, castration is indirectly associated with BRD via measurements of ADG, acute phase protein, or cortisol concentrations

(Chase et al., 1995; Fischer et al., 2001; Pinchak et al., 2004). However, ADG is consistently reduced upon delayed castration (i.e., castration of yearling-age bulls on arrival to the feedlot), suggesting that it is a stressful event (Zwelacher et al., 1979; Brazle, 1992; Faulkner et al., 1992; Chase et al., 1995; Berry et al., 2001; Fischer et al., 2001; Pinchak et al., 2004). Given the immunosuppressive impacts of chronic stress, it is plausible that cattle castrated upon arrival to the feedlot are more susceptible to disease. Bulls may also be at greater risk for BRD because previous management or vaccination procedures on the ranch of origin is likely less than that of steer cohorts (Richeson et al., 2013).

Similarly, dehorning is conducted upon received cattle if necessary, and studies have reported greater disease incidence and/or decreased performance in groups of cattle that are dehorned on arrival (Martin et al., 1982; Loxton et al., 1982; Hand and Goonewardene, 1989; Goonewardene and Hand, 1991). Without question, it is still necessary to castrate and dehorn cattle for best management practices in the feedlot. Currently, research is being conducted to better understand how and when to implement these practices to reach utmost performance and welfare of the animal.

Calf origin

Numerous studies have demonstrated that newly received cattle are at greatest risk for BRD (Mac Vean et al., 1986; Alexander et al., 1989; Ribble et al., 1995; Sanderson et al., 2008). Further, cattle purchased from auction markets are at increased risk for BRD than those which are direct marketed from their ranch of origin (Gummow and Mapham, 2000; Step et al., 2008), which has been described previously in this dissertation. This is likely due to factors commonly associated with calves marketed

through auction markets, such as commingling and weaning (Martin et al., 1978; Alexander et al., 1989; Ribble et al., 1995; Sanderson et al., 2008).

It is clear that multiple predisposing factors confound one another and may work additively to increase an animal's susceptibility to BRD. In addition to these predisposing factors, naturally occurring genetic variations between individual animals, due to gender, breed, or temperament exists, resulting in differences in the immunological consequence of these stressors (Hughes et al., 2013; Appendix). The complexity of BRD illustrates the need for research related to management strategies that could potentially mitigate the insult of certain predisposing factors.

Vaccination Strategies in the Feedlot

Types of Respiratory Vaccines

Given that the immune system is altered when cattle experience chronic stress and the concurrent risk for pathogen exposure during transition to the feedlot, it is necessary to evaluate current vaccination strategies for the prevention of BRD in order to investigate areas that may be improved. Respiratory vaccines used in the feedlot may contain viral or bacterial components or a combination of both.

Viral respiratory vaccines

With regard to BRD prevention, viral vaccine antigens typically consist of some combination of the implicated viruses mentioned previously, and the agents used in commercial vaccines are killed or modified-live viral versions. Killed vaccines include viral components that have been rendered non-infectious, often via chemical treatment such as formaldehyde. The killed vaccines require a highly immunogenic adjuvant, as well as a booster vaccination, to be effective at stimulating a protective immune response

(Oirshot et al., 1999). The major advantage of killed vaccines is their increased safety and stability. Conversely, modified-live vaccines contain attenuated viral strains which replicate within the host cells, resulting in an immune response like that which occurs following natural exposure to a virus. Typically, the viral agent is passed through multiple cell cultures to attenuate the virus so that it stimulates immunity without causing clinical disease in the animal (Lobmann et al., 1984). Modified-live respiratory vaccines are more commonly used in the feedlot because they do not require a booster and the duration of immunity is longer than that of a killed vaccine (Griffin and Smith, 2007).

Until the late 1990s, vaccinating against viral agents alone was the approach to prevention of BRD in the feedlot (Hjerpe, 1990; Thomson et al., 1993); however, the efficacy of this procedure is questionable due to the likelihood of preexisting viral infection among newly received cattle (Hjerpe, 1990; Griffin et al., 1995; Wittum et al., 1996). Nevertheless, feedlots continue to vaccinate calves on arrival with viral vaccines. According to the USDA (Feedlot 2011 report), 96.6% of feedlots vaccinate for BVDV, 93.7% for BHV-1, 85.1% for PI-3V, and 89.5% for BRSV. More recently, the incorporation of *M. haemolytica* and *P. multocida* bacterins to feedlot on-arrival vaccination strategies has been implemented.

Bacterins

Similar to viral respiratory vaccines, bacterins contain either killed or avirulent-live bacterial components, along with distinct leukotoxoid fractions, with the goal of stimulating a protective immune response. The use of bacterins in the feedlot has increased, with two out of three feedlots reporting use of combination vaccines which utilize the two most common bacterial agents associated with BRD: *M. haemolytica* and

P. multocida (USDA – APHIS, 2011). Bacterins have been manufactured for over a century (Mosier et al., 1989), and early products were developed from live cultures of *Bacillus boviseptica*, which has since been divided into *M. haemolytica* and *P. multocida*. These early bacterins were ineffective (Miller et al., 1927) or detrimental (Farley, 1932). In more recent studies, *M. haemolytica* bacterins have demonstrated no benefit (Hamdy et al., 1965; Martin, 1983) or were detrimental (Schipper and Kelling, 1971; Friend et al., 1977; Wilkie et al., 1980).

The importance of Lkt in the pathogenesis of *M. haemolytica* infection was later discovered (Shewen and Wilkie, 1982, 1985; Gentry et al., 1985) and commercial bacterins were developed containing cell-free *M. haemolytica* A1 culture supernatant, which contains the Lkt produced during logarithmic-phase growth of cell culture (Shewen and Wilkie, 1988; Shewen et al., 1988). These bacterins are considered "subunit-enriched" vaccines because they capitalize on various subunits associated with *M. haemolytica* logarithmic-phase growth. Experimental trials using these Lkt-containing bacterins demonstrated improved efficacy over previous bacterin products (Shewen et al., 1988; Bateman, 1988; Jim et al., 1988; Thorlakson et al., 1990).

Since then, various pharmaceutical companies have developed similar subunit bacterins which consist of various outer membrane extracts (Srinand et al., 1996), including toxoids, which contain toxins secreted by bacteria that have been inactivated via formalin treatment. The inactivated toxin is safe in the host, yet stimulates active immunity against virulent Lkt for future exposure (WHO, 2015). Additionally, combination parenteral vaccines exist which contain both viral and bacterial components in a single dose, and various combinations exist for producers to choose from. The

combination vaccines evaluated in Chapters IV and V of this dissertation each contain unique Lkt-antibody inducing factors as their bacterial components.

Vaccination upon Arrival to the Feedlot

As previously mentioned, the feedlot industry has been administering viral vaccines to cattle on arrival for decades, with many recently beginning to adopt the use of bacterins (Bowland and Shewen, 2000). According to the USDA-APHIS Feedlot Report (2011), use of vaccines containing bacterial agents is more common in feedlot operations with a smaller capacity of 1000 to 7,999 head (66.1% of feedlots surveyed), compared to feedlots with a capacity of 8,000 head or more (58.2% of feedlots surveyed).

When discussing vaccines, it is important to understand the difference between vaccine efficacy and vaccine efficiency. Vaccine efficacy can be defined as the percent reduction in disease incidence in a vaccinated group compared to an unvaccinated group under optimal conditions, where subjects are healthy and unstressed. Efficacy is measured using controlled studies where a pathogen challenge is administered, followed by objective measurements of immune stimulation. Vaccine efficiency, or effectiveness, describes the ability of a vaccine to prevent outcomes of interest in the "real world". Multiple factors impact the ability to assess vaccine efficiency, including sample size, pathogen load, and animal characteristics. While efficacy is certainly vital, monitoring vaccine efficiency is crucial in the development and justification for use of vaccines (McNeil, 2015).

The difficulty in extrapolating efficacy of vaccines to the commercial production setting lies in the fact that vaccines are often tested in "clean" cattle; those with no parasites, little stress, and no existing disease. Realistically, conditions are much less

ideal in cattle arriving to the feedlot, making it difficult to interpret how well a vaccine might stimulate immunity (Wren, 2011) and provide a measurable reduction in clinical BRD in the commercial feedlot setting.

Vaccine failure

When an animal does not mount an immune response sufficient to protect it from clinical disease, this is considered vaccine failure. Multiple factors can inhibit an animal's response to vaccination. In young beef calves, antibody interference from passively acquired antibodies in colostrum may inhibit the humoral immune response to vaccination, but a recent study suggests significant BVDV-specific cellular and antibody responses in beef calves with maternal antibodies present (Powell et al., 2011). If antibodies from colostrum are present at sufficiently high titers upon vaccination, they may reduce binding of vaccine components to susceptible cells/receptors, preventing the viral replication or bacterial colonization needed to stimulate the production of antibodies for future protection (Zhang and Finn, 2004). Passively acquired antibodies are typically present three to six months, and this phenomenon is referred to as passive or maternal antibody interference (Griffin and Smith, 2007). Other factors may inhibit an animal's response to vaccination such as stress-induced immunosuppression during transition to the feedlot, concurrent infection with wild-type pathogens at the time of vaccination, severe parasitism at the time of vaccination, or improper handling and administration of the vaccine (Wren, 2011).

Most vaccines, when administered properly and under the right conditions, are efficacious due to the rigorous testing that occurs before they reach the market. However, in cattle experiencing numerous stressors, vaccination may elicit a

physiological reaction known as vaccine "sweats", which includes depression and slight fever (Adriaan et al., 2003). This phenomenon occurs due to release of pro-inflammatory cytokines (i.e., IL-1, IL-6, TNF-alpha) in response to vaccination, which are produced by leukocytes at the site of injection in order to stimulate an immune cascade that eventually leads to antibody protection against the antigens contained in the vaccine. If cytokines become systemic and breach the blood-brain barrier, appetite is reduced and fever is induced (Bagley, 2006). Cattle receiving multiple vaccine antigens upon arrival have a greater chance to experience vaccine sweat because of greater circulating concentrations of cytokines. The amount of circulating immune effectors will differ, depending on type of vaccines used: killed vs. modified-live viral vaccines. Live-attenuated vaccines are considered more immunogenic than killed vaccines because they induce activation of the innate immune system to the extent that natural infection is mimicked and viral replication occurs (World Health Organization, 1967). Conversely, killed vaccines do not elicit microbial replication, making them more limited in their ability to activate innate immune responses; however, consequently, they are considered safer because they do not induce vaccine sweats (van Duin et al., 2006).

Field-based vaccine data

Difficulty exists in assessing the benefit of vaccination of calves upon arrival to the feedlot through existing research publications, and much of the evidence is anecdotal. Often, cattle arrive to the feedlot after being exposed to respiratory pathogens during movement through the marketing channel and with a compromised immune system, thus the capability of a vaccine to elicit protection after arrival is probably limited (Fulton, 2009). Conflicting results most likely occur because of variation of cattle and conditions

between studies, in addition to the complexity of the disease. Kimman et al. (1989) reported severe BRSV infection in calves two d after vaccination with a modified-live BRSV vaccine. Serum BRSV-specific IgM antibodies in non-vaccinated cattle indicated that BRSV was present among the herd two d prior to vaccination, suggesting that natural infection was enhanced by the vaccine. This study demonstrates the potential negative implications of vaccinating calves concurrent with natural viral infection. MacGregor et al. (2003) observed no differences in BRD mortality, morbidity, or performance between high-risk calves administered an *M. haemolytica* vaccine and non-vaccinated controls, suggesting that vaccination on arrival with a bacterin provided no detectable benefit. However, multiple studies have demonstrated effectiveness of vaccination with multivalent vaccines, evidenced by a greater ability to withstand viral challenge (Valla, 1986; Morisse et al., 1990; Cravens, 1991; Hansen et al., 1992; More O'Ferrall, 1993; Catalan et al., 1994; Peters et al., 2004); yet, these are controlled studies in which conditions are ideal at the initiation of the study and not representative of actual field conditions.

Why does BRD continue to plague the feedlot industry?

With regard to immune status at time of arrival to the feedlot, Fulton et al. (2002a) observed improved immunity among calves entering the feedlot with high antibody titers to 4 infectious agents, and other studies (Martin et al., 1989; Martin et al., 1999; Booker et al., 1999; O'Connor et al., 2001) have demonstrated greater protection against BRD in calves with high titers of BVDV-specific antibody. These data suggest that cattle arriving to the feedlot, having been previously vaccinated (or exposed to wild type virus), have a greater chance to remain healthy and maximize production efficiency.

In spite of this notion, many believe there is potentially greater profit in managing disease in high-risk cattle than paying a higher price for healthy calves, hence the persistence of BRD incidence in the feedlot. Controlling BRD, rather than preventing it through management and vaccination, has been made more feasible than in the past due to the use of antimicrobial metaphylaxis. Metaphylaxis is the use of antibiotics to control BRD outbreak in calves considered "high risk" before the clinical signs of bacterial infection are evident (Fulton, 2009). Additionally, from the cow-calf producer perspective, it can be difficult and lack incentive to precondition, as was described earlier.

As BRD continues to be a significant issue in the cattle industry, a need exists to further explore strategies to make vaccination more effective at the feedlot sector, in addition to creating easier means of vaccine administration for those at the cow-calf sector. While it is difficult to assess the efficacy of vaccines under all commercial production conditions, with all manner of vaccine products containing various combinations of antigens, it is certain that animals that arrive healthy to the feedlot and have been vaccinated have greater ability to withstand disease. One possible avenue to explore with regard to easier vaccination administration is the mucosal route.

Mucosal Immunity

Mucosal vs. Systemic Immunity

The vaccines most commonly administered to cattle, are done so via the parenteral route, meaning they are administered either subcutaneously or intramuscularly. This primarily induces a systemic proinflammatory response within the body, eventually stimulating the production of humoral antibodies from antigen-stimulated B cells (i.e., plasma cells), which neutralize specific antigens or promote the phagocytosis of bacteria

and inactivation of their toxins, or viruses. Therefore, systemic immunity simply refers to immune responses that occur within the body, which are initiated in the various lymph nodes and spleen following entry of antigens to the internal organs or blood (Rayevskaya and Frankel, 2001). Conversely, the mucosal immune system describes a compartment of relatively greater size which is located near the epithelial surfaces where most pathogens actually invade a host. The only physical barrier protecting the internal body from potential pathogen invasion is a thin layer of mucosal epithelium. The mucosal surface requires protection which exists in the form of cells and molecules that comprise the mucosal immune system (Brandtzaeg, 2009).

The mucosal immune system is comprised of all the body surfaces lined with mucus-secreting epithelium: the esophagus and gastrointestinal tract, the upper and lower respiratory tract, and the urogenital tract. The exocrine glands associated with these organs are also involved and include the conjunctivae and lachrymal glands of the eye, the salivary glands, and the lactating breast. These surfaces act as portals of entry for an array of foreign antigens that may or may not be pathogenic. For example, the host ingests enormous quantities of food proteins, which in most circumstances do not harm the host. Additionally, mucosal surfaces of the respiratory and gastrointestinal tracts contain a plethora of bacteria which live in symbiosis with the host, known as commensal bacteria. The host's mucosal immune system has the ability to distinguish between harmful pathogens and antigens in food and the natural microbiota (Kunisawa et al., 2008). This array of components forms the largest mammalian lymphoid organ system, known as the mucosa-associated lymphoid tissues (MALT; Mestecky, 2005).

Mucosal immune responses

The mucosal immune system has three main functions: 1) to protect the mucous membranes against colonization and invasion by potentially dangerous microbes, 2) to prevent uptake of undegraded antigens including foreign proteins derived from ingested food, airborne antigens, and commensal microorganisms, and 3) to prevent the development of potentially harmful immune responses to antigens if they reach the host's interior (Holmgren and Czerkinsky, 2005). Within the MALT, epithelial cells and membrane, or "M" cells, sample antigens via absorption and transport them to antigen-presenting cells (APC). These APC may be dendritic cells (DCs), B lymphocytes, or macrophages, which then present the antigen to conventional CD4+ and CD8+ T cells, all located in the inductive sites. The APC involved is dependent upon antigen type, and in the case of entry of harmless antigens such as food proteins, mucosal DCs generate T helper 2 (Th2) cell responses and actively suppress systemic immunity; a phenomenon known as "oral tolerance". Conversely, those antigens perceived as deleterious instigate effector immune responses and engage the humoral and cellular components of immunity (Iwaski and Kelsall, 1999; Mowat, 2003; Bilsborough and Viney, 2004;). The primary antibody involved in the adaptive humoral defense at mucosal surfaces is secretory IgA (sIgA). With regard to BRD, natural pathogen entry occurs when the mucosal tissue of the nasal and oral surfaces are breached, which stimulates the production of sIgA in mucosal tissues; therefore, investigating the mucosal route of immunization is of interest (Gerdtz et al., 2006).

The Role of Secretory IgA

Bodily fluids including tears, nasal secretions, and saliva contain antibodies; specifically, sIgA. Secretory IgA was first isolated and characterized in 1960 by Joseph F. Heremans (Tomasi, 1992). Other researchers during this same time period demonstrated the predominance of sIgA in saliva and several other secretions in humans. More recently, sIgA has been considered the first line of defense in protecting mucosal epithelium from toxins and pathogens. Activation of sIgA is dependent upon the antigen presentation described previously, which ultimately leads to B-cell class switching in the MALT (He, 2007; Brandtzaeg, 2010). Multiple cytokines are also involved in stimulating the production of sIgA, including IL-4, transforming growth factor-β, IL-5, IL-6, and IL-10.

The structure of sIgA is almost exclusively a polymer, usually a dimer, in which two immunoglobulin monomers are linked by a joining chain. During formation of the antibody, cleavage leads to a secretory component on the structure, giving the antibody its name (Cerutti, 2010). The role of sIgA is complex and has not been fully elucidated. It is known to down regulate proinflammatory responses normally associated with the uptake of highly pathogenic bacteria and allergenic antigens, in addition to promoting the transport of antigens across the mucosal epithelium to DC. Importantly, it plays a crucial role in maintaining the homeostatic balance between the host and commensal organisms, which, in the context of this paper, pertains to commensal bacteria implicated in BRD. Finally, the secretory component of the antibody allows it to neutralize toxins or enzymes in the MALT (Fagarasan et al., 2010). The role of sIgA in mucosal tissues is vital to

understand when considering novel vaccination routes in cattle. When administering a vaccine antigen via either the nasal or oral route, it is necessary that the antigen not create "tolerance", so that an immune response will be mounted to the antigen. To achieve this, various strategies are used to ensure immunogenicity of mucosal vaccines.

Strategies for Mucosal Vaccine Delivery

Developing mucosal vaccines that are safe, effective, and cost effective has proven to be a difficult task. Multiple delivery systems have been evaluated and include live vectors, microparticles, and liposomes.

Live vectors

For both humans and animals, live viral and bacterial vectors have been developed for enhancing vaccine antigen recognition. Live vectors operate on the same principal as live-attenuated viral vaccines. Ideally, a live vector possesses the following features: 1) non-pathogenic to animals and humans, 2) easy to manipulate, 3) easy and cost-effective to produce, 4) contain stable genome, 5) contain well defined sites for insertion of foreign genes, 6) easy to deliver, 7) no integration into host genome, and 8) induce both mucosal and systemic immune responses when delivered orally or intranasally. Currently, live vector mucosal vaccines exist for multiple viral diseases including Rabies, Distemper, and Equine flu to name a few (Gerdts et al., 2006).

Bacterial vectors

Live bacterial vectors offer advantages over current bacterial agents because they target specific APC via bacterial secretion systems in the mucosal tissue (Autenrieth and Schmidt, 2000; Gentschev et al., 1996; Gentschev et al., 2002). Multiple bacterial vectors carry plasmid DNA across the mucosal surfaces (Darji et al., 1995; Dietrich et al.,

2000; Loessner and Weiss, 2004; Sizemore et al., 1997), with the first invented vectors being *Salmonella* and *Shigella* spp. (Stocker, 2000). Relatively, bacterial vectors are inexpensive to develop and produce, can be delivered either orally or intranasally, can include multiple foreign genes to protect the host against multiple pathogens simultaneously, can induce humoral and cellular immune responses, and can be controlled with antibiotics if adverse effects arise. One of the largest concerns with using bacterial vectors, since they are from attenuated strains, is the potential for reversion to virulence within the host (Medina and Guzman, 2001). Additional research is needed in this area to determine if bacterial vectors elicit a robust immune response sufficient to protect the host, and currently there are not many reports of these being applied in veterinary species (Gerdts et al., 2006).

Viral vectors

Viral vectors may also be an effective way to deliver vaccine antigens across mucosal surfaces (Sheppard, 1999; Yokoyama et al., 1998). When a virus is used as a vector, it serves as a carrier for another pathogen (e.g., viral or otherwise), making it a similar strategy to that of an adjuvant. Poxviruses, herpesviruses, and adenoviruses have all been used as viral vectors in veterinary vaccines to stimulate immunity of the host to various other pathogens (Yokoyama et al., 1998). Typically, these vectors are made up of genetically modified viruses which will undergo the proper folding and processing of antigen within the host (Sheppard, 1999; Yokoyama et al., 1998). Several viral vectors have been evaluated for use in mucosal vaccines. Kit et al. (1991) used BHV-1 to vaccinate young calves for foot and mouth disease virus (FMDV) by delivering the viral protein of FMDV. Likewise, Kweon et al. (1999) vaccinated calves for BVDV using

BHV-1 as a vector to deliver the glycoprotein E2 of BVDV. Bovine herpesvirus-1 was also administered to calves to induce protection against BRSV (Schrijver et al., 1997). While there seems to be potential for viral vectors in veterinary practice, specifically with respect to BRD viruses, various concerns exist that need more research to substantiate. Safety and stability of these vaccines, their applicability, and their effectiveness in the presence of existing immunity all need to be further investigated (Gerdts et al., 2006).

Particulate Delivery Systems

Bacterial or viral agents that are administered in their killed form, and therefore considered "non-replicating", are not effectively immunogenic when administered mucosally. Sufficient uptake of antigens by APC is necessary to stimulate a mucosal immune response and, again, the MALT is accustomed to tolerating a plethora of foreign bodies which are harmless to the host. One potential way to overcome this barrier is via particulate delivery systems which may significantly improve the immunogenicity of antigens in the MALT. This area has only recently been studied, yet there appears to be large potential for particulate delivery systems to improve the safety and efficacy of mucosally delivered antigens (Gerdts et al., 2006).

Microparticles

The primary goal of encapsulating antigens within microparticles is to protect the antigen from degradation and increase uptake of antigens into specialized MALT (O'Hagan, 1996). Mast cells in the follicle-associated epithelium are predominantly responsible for the uptake of microparticles (Beier and Gebert, 1998; Bowersock et al., 1999). This uptake of microparticles is often poor and is the major limitation of this delivery mechanism because it results in suboptimal immune responses. Incorporating

specific types of adjuvants, which will be discussed later, can improve the efficacy of these vectors (Foster et al., 1998).

Examples of polymers used as microparticles are poly-lactide-co-glycolide (PLG), alginate, polyphosphazenes, and starch. Use of PLG microparticles has been successful at enhancing immunological responses in multiple vaccine studies (O'Hagan, 1998; Vajdy and O'Hagan, 2001). O'Hagen (1998) reported increase serum IgG and intestinal IgA in response to oral immunization of mice with ovalbumin encapsulated in PLG microparticles. Additionally, mice which were mucosally immunized with PLG microparticle vaccines were better protected against an oral challenge with *S. typhimurium* compared to those immunized with antigen in Freund's adjuvant (Allaoui-Attarki et al., 1997; Fattal et al., 2002). Interestingly, in mice challenged with *Bordetella pertussis* intranasally, those receiving a single oral immunization with fimbriae from *Bordetella pertussis* encapsulated in PLG microparticles were protected against clinical disease, indicating that the oral delivery system stimulated the common mucosal system to protect against infection at a distant mucosal site comprising the naris (Jones et al., 1996). This phenomenon, in which immunity in distant mucosal tissues is activated by stimulated immunocytes in remote mucosal sites, is referred to as mucosal "crosstalk" (Quiding et al., 1991; Kozlowski et al., 1997; Eriksson et al., 1998). Exploiting the mucosal crosstalk mechanism could be advantageous in the development of an oral vaccine for cattle, to stimulate distant mucosal tissues of the nasopharynx.

Multiple other studies in mice have demonstrated the efficacy of PLG microparticles in the face of pathogenic challenge (Chen et alk., 1998; Herrmann et al., 1999; Singh et al., 2001; Carcaboso et al., 2004). Conversely, a study in pigs reported no

significant antibody response following PLG immunization (Felder et al., 2000); yet, injection of 2-week-old chicks with PLG microparticles containing *S. enteritidis* produced a detectable sIgA antibody response and protected against oral and intramuscular challenge (Liu et al., 2001).

Another microparticle used in mucosal vaccination trials is alginate, which is a naturally occurring carbohydrate from kelp (Gerdts et al., 2006). This particle has been tested in several studies using cattle. Oral immunization of cattle with ovalbumin in alginate microparticles stimulated IgA and IgG responses in the respiratory tract (Bowersock et al., 1998), demonstrating that stimulating one component of the MALT can contribute to immune stimulation in other areas (i.e., stimulating oral mucosa, which translates to the nasal mucosa). Antibodies in serum, nasal secretions, and saliva of cattle were produced following intranasal immunization with porcine serum albumin in alginate microparticles; however, oral immunization did not elicit a detectable immune response, suggesting that intranasal administration was more effective for inducing immunity in the nasal mucosa (Rebelatto et al., 2001). Studies in rabbits (Bowersock et al., 1999) and mice (Kidane et al., 2001) have also demonstrated efficacy of alginate microparticles for mucosal delivery of vaccine antigen.

Immunostimulating complex

A second particulate delivery system is a small 40 nm nanoparticle composed of saponin, lipids, and antigen called immunostimulating complex, better known as ISCOM. The adjuvant activity of saponin within the particle, in addition to ISCOM's ability to target and recruit APC, makes it a viable particulate component of mucosal vaccines (Morein and Hu, 2004). The most effective mechanism that ISCOM possesses is the

ability to stimulate innate immunity and the production of cytokines such as IL-12 (Furrie et al., 2002; Smith et al., 1999). Parenteral veterinary vaccines now contain ISCOM because of its ability to enhance immune responses to a variety of antigens in laboratory and domestic animals (Bowersock et al., 1999; Morein and Hu, 2004).

Multiple studies in mice demonstrate induction of mucosal IgA, Th1 and Th2 cell responses, recruitment of DC and macrophages, and recruitment of B cells following ISCOM immunization (Maloy et al., 1995; Mowat et al., 1999; Furrie et al., 2002; Lovgren et al., 1990; Hu et al., 1998). In dogs, high mucosal antibody responses following ISCOM immunization were reported in the absence of systemic antibody responses (Carol and Nieto, 1998). Further research in domestic animals is warranted to fully explore this technology.

Liposomes

Liposome is a phospholipid vesicle which increases antigen uptake and presentation by APC (Gregoriadis, 1990). Extensive research of liposome administration in mice, either orally or intranasally, has revealed a tendency for tolerance of the host with this particular mechanism (Zho and Neutra, 2002). To remove this barrier, liposomes have been conjugated to other proteins or delivered alongside adjuvants, improving the mucosal immune response (Harokopakis et al., 1998). In studies where adjuvants were incorporated into liposome containing vaccines, efficacy was drastically improved (Zhou et al., 1995; Tana Watari et al., 2003; Baca-Estrada et al., 2000; Wang et al., 2004; Fukutome et al., 2001).

Each of these particulate agents demonstrates potential for inducing mucosal immunity. Research models using larger animal species, such as cattle, is warranted

because much of the existing body of literature contains laboratory-based research in mice. The use of adjuvants alongside these different immunostimulatory particles can be important in aiding an appropriate immune response to enhance protection against certain antigens.

Adjuvants

Adjuvants have been used to aid in vaccine efficacy since the 1920s (Glenny et al., 1926; Lewis and Loomis, 1924). These substances are administered alongside vaccine antigens to enhance the immune response, which they accomplish in five distinct methods: 1) Immunomodulation – typically this involves the upregulation of cytokines (Cherwinski et al., 1987; Mosmann and Moore, 1991; Romagnani, 1991), 2) Presentation – antigen presentation to DC, Langerhans cells (LC), and macrophages, which leads to increased neutralizing antibody response (Brewer et al., 1994), 3) Induction of CD8+ cytotoxic T-lymphocyte (CTL) responses – aids in the processing of protein yielding correct class 1 restricted peptides (Neefjes and Momburg, 1993), 4) Targeting – targets lectin receptors on macrophages and DCs (Brewer et al,. 1994), and 5) Depot generation – short- or long-term depots are generated which lead to a more gradual release of vaccine antigens (Freund, 1951).Numerous types of adjuvants exist, and relevant adjuvants pertaining to the context of this dissertation will be discussed.

Water-in-oil emulsions

Water-in-oil emulsion adjuvants consist of microdroplets of water, stabilized by surfactant in a continuous oil phase which is typically mineral oil, squalene, or squalane (Freund, 1951; Salk and Laurent, 1952). The most common water-in-oil adjuvant is Freunds incomplete adjuvant (FIA) and has been used in human and veterinary medicine

for many decades (Firehammer and Berg, 1966). Today, FIA is largely discredited as an effective adjuvant because of its poor immunogenicity, though it provides a desirable short term depot, is inexpensive and simple to formulate, and induces antibody responses for hydrophilic immunogens. When soluble immunomodulators can be incorporated with water-in-oil emulsions they can be useful (Cox and Coulter, 1997). Metastim is a proprietary adjuvant used in one of the vaccines evaluated in Chapter IV of this dissertation and is a water-in-oil emulsion. The properties of Metastim and their potential impact on intranasal vaccine efficacy will be discussed in further detail in Chapter 4.

Oil-in-water emulsions

In contrast to the above, oil-in-water emulsions consist of microdroplets of oil, stabilized by a surfactant, in a continuous water phase (Allison and Byars, 1992). These adjuvants offer excellent antigen presentation and moderate targeting. Additionally, they are inexpensive, safe (Ott et al., 1995), and excellent for use where lipophilic immunomodulators can be incorporated.

Water-in-oil-in-water emulsions

Finally, water-in-oil-in-water emulsions consist of microdroplets of water in oil, dispersed through an aqueous phase. These adjuvants function as an intermediate, releasing antigens more rapidly than water-in-oil emulsions, but more slowly than oil-in-water emulsions (Aucouturier et al., 2001). Short and long-term immunity can be achieved with use of this adjuvant; however, these emulsions are not commonly used because of issues with stability; however, newer techniques have lessened this concern (Macy, 1997).

Opportunities and Challenges with Mucosal Vaccination of Cattle

As previously mentioned, vaccines delivered to cattle are typically parenteral and primarily elicit systemic immune responses, rather than mucosal. However, the route of mucosal vaccination against BRD is of interest because the initiation of the disease begins with entry across the mucosal tissues of the respiratory tract (Hodgins et al., 2005). Aside from the need for immunity against respiratory viruses at the mucosal surface, the ease of delivery, and the absence of injection site lesions or reactions are non-immunological justification to further research this area (Roeber et al., 2002). In food producing animals, this could mean administering vaccine via the feed or water, which would lead to less stress on the animals and significantly less labor required by livestock producers. Furthermore, carcass defects resulting from injection site lesions would be reduced with the use of mucosal vaccination.

While it appears that mucosal vaccination could enhance protection and mitigate issues related to BRD, there are numerous inherent challenges facing mucosal antigen delivery. Regarding orally administered vaccines, cattle are particularly challenging since they are ruminant animals. Bowersock et al. (1999) used microspheres that resist antigen breakdown in the rumen as means to stimulate the Peyer's patches of the small intestine via oral delivery of ovalbumin as antigen. Another possible approach to oral vaccination is to exploit the process of rumination or "cudding", by allowing encapsulated antigen in the rumen to repeatedly contact pharyngeal lymphoid tissue during rumination.

Shewen et al. (2009) fed transgenic alfalfa, which expressed a truncated form of Lkt (i.e., Lkt 50; Lee et al., 2001) to five-month-old calves each d for 5 d. Three weeks

after the second feeding, calves were challenged by intrabronchial administration of *M. haemolytica* to elicit infection sufficient to induce a recall immune response, but not produce pneumonia in controls. Post-challenge, calves were euthanized for collection of mononuclear cells from blood, tonsil, spleen, and lymph nodes. Serum and nasal swabs were also collected at various time points throughout the trial. An increase in Lkt specific IgA in nasal secretions was observed 1 week following the second feeding; however, this increase was transient and no differences between vaccinates and controls were observed at the time of *M. haemolytica* challenge. Additionally, no changes in serum antibodies were observed, indicating that systemic immunity was not stimulated by the transgenic feed method. Interestingly, in a similar pilot study conducted by Shewen et al. (2009), the *M. haemolytica* challenge used was sufficient to cause clinical pneumonia in the two control calves (lesion score 5 out of 10 for both, 11% and 27% pneumonic tissue), yet the two calves fed transgenic alfalfa had no clinical signs of pneumonia or pulmonary lesions at necropsy. It is important to be cautious in interpreting this as vaccine efficacy, given the very small number of subjects used in the study.

Administering vaccine to cattle orally could be a favorable method of mucosal vaccine administration; however, in neonatal calves that are not yet ruminating, intranasal administration may be more effective, since the principal behind oral vaccination is primarily based on antigen recognition in the gut mucosal tissues. Not only does intranasal vaccination lend to the ease of delivery, it also targets the nasopharyngeal lymphoid tissue directly. Difficulty lies in administering vaccines to young calves during the period when passive immunity from the dam may interfere with the active immune

response (Prado et al., 2006). Hodgins and Shewen (1998) demonstrated that calves parenterally vaccinated with a commercial *M. haemolytica* bacterin prior to 6 weeks of age did not produce Lkt neutralizing antibodies. Potential to circumvent this issue exists in the use of adjuvants, such as ISCOM, discussed previously. Shewen et al. (2009) used ISCOM-containing *M. haemolytica* bacterin to vaccinate colostrum-fed dairy heifers at 4 and 6 weeks of age, either intranasally or subcutaneously. The intranasal route resulted in increases in Lkt-specific IgA titers in nasal secretions 1 week following the first vaccination at 4 weeks of age, suggesting that this mucosal vaccine overcame the maternal antibody blockade.

These oral and intransal studies illustrate the potential for mucosal vaccinations to stimulate immunity in cattle and a need for further research which investigates the use of mucosally delivered vaccine antigens implicated in BRD. Currently, an intranasal vaccine for the prevention of BRD caused by BRSV, BHV-1 and PI-3 exists on the market and is safe for use in calves as young 3 d old (Zoetis Technical Bulletin, 2010). This vaccine (Inforce 3, Zoetis, Kalamazoo, MI) is the only intranasal vaccine to be granted a USDA label claim for prevention of disease caused by BRSV. Notably, this vaccine does not contain BVDV, which is a key respiratory virus implicated in BRD, and a commercial mucosal vaccine containing BVDV antigen(s) does not currently exist.

Conclusion

Although year to year variability exists, BRD prevalence in the feedlot has not changed significantly since the current beef production system was implemented in the 1960s. The segmented structure of the US beef production system contributes to issues in disease manifestation and management. Currently, the time, labor, and financial

incentive are not sufficient for most small cow-calf producers to precondition their calves prior to marketing, resulting in a significant proportion of unvaccinated and poorly managed calves entering the feedlot. Additionally, the stress of abrupt weaning, transport, social reorganization, and poor nutrition during the transition to the feedlot impair immune function and potentially hinder the efficacy of vaccination for respiratory viruses and bacteria upon arrival to the feedlot. Further, there is little evidence demonstrating that respiratory vaccination of cattle upon arrival to the feedlot is able to clearly mitigate clinical BRD.

Novel vaccination strategies, such as the use of oral or intransal mucosal vaccines, provide potential to change the way disease is prevented in the beef and dairy industry. A vaccine that cow-calf producers could more easily administer to calves prior to marketing could improve the adoption of respiratory vaccination before sale, and thus the health of cattle entering the feedlot. Numerous challenges exist with mucosal vaccination, including the complex nature of BRD, difficulty in finding balance between under- and over-stimulation of immunity, and the cost to produce effective adjuvants or delivery mechanisms. As BRD continues to be a major health issue in the feedlot industry, and the public becomes more concerned with animal welfare and use of antibiotics in animal agriculture, it will be necessary to continue research efforts which investigate the potential for mucosal vaccination strategies to prevent BRD.

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CHAPTER II

A PROOF-OF-CONCEPT STUDY TO EXPLORE SAFETY AND EFFICACY OF ORAL MODIFIED-LIVE VIRUS RESPIRATORY VACCINE ADMINISTRATION IN BEEF CALVES

H.D. Hughes

West Texas A&M University

ABSTRACT

A total of 42 (n = 10 or 6/treatment) clinically healthy, previously unvaccinated crossbred beef steer calves were stratified by pre-trial serum BVDV type 1a antibody titer concentration, then assigned randomly to 1 of 5 vaccination treatment regimens applied on d 0: 1) 1 × dose oral – pentavalent MLV respiratory vaccine (OV1; 2 mL Pyramid® 5 without Metastim administered in the buccal cavity), 2) 5 × dose oral – pentavalent MLV respiratory vaccine (OV5; 10 mL Pyramid® 5 without Metastim administered in the buccal cavity), 3) 10 × dose oral – pentavalent MLV respiratory vaccine (OV10; 20 mL Pyramid® 5 without Metastim administered in the buccal cavity), 4) positive control (PCON; 2 mL Pyramid® 5 with Metastim administered s.c., in the neck), 5) negative control (NCON; 2 mL sterile saline administered in the buccal cavity). The positive control group had a greater BRSV-specific serum antibody concentration when compared to negative control on d 28 ($P = 0.04$). No differences in the percent positivity of BRSV-specific secretory IgA from oral ($P = 0.68$) or nasal ($P \geq 0.07$) swab samples were observed among treatments. A marked increase in basophils was observed for OV10 on d 2 ($P < 0.001$). Real time PCR results indicated post-vaccination nasal shedding of PI3V in oral vaccinates only; whereas, BRSV was detected on d 0 for all treatments indicating unwanted wild type exposure to this particular virus before trial initiaition. Results from this study indicate no clear immunological benefit to oral vaccination of calves using Pyramid® 5, without adjuvant (Metastim). Future research examining

immunological responses after administration of MLV respiratory vaccine directly into the gastrointestinal tract or oral adminstration alongside a particulate delivery system or live vector could be beneficial.

Introduction

Most viral and bacterial pathogens enter a host through mucosal surfaces, and mucosa-associated lymphoid tissue (MALT) of the respiratory tract is a critical mechanism for respiratory pathogen entry and immune defense when exposure via the nose and/or mouth occurs. In cattle, vaccinations against respiratory disease are typically administered parenterally, which does not effectively stimulate immunity at mucosal surfaces; whereas, evidence indicates that vaccination via the mucosal (i.e., intranasal) route stimulates immunity at the MALT, in addition to systemic immunity (Muir et al., 1994; Mowat and Viney, 1997; Bowersock et al., 1999; Xiang et al., 1999). Inductive sites in the MALT, such as gut-associated lymphoid tissue in the intestines, take up an antigen and present it to antigen-specific B lymphocytes. These antigen-specific cells elicit the production of secretory IgA (sIgA). Secretory IgA-producing lymphocytes may also migrate to other mucosal sites in the body; a phenomenon known as "mucosal crosstalk" (McDermott and Bienenstock, 1979). Oral administration of antigen is capable of stimulating mucosal immunity in the respiratory tract, either via direct contact of antigen or via mucosal crosstalk (Mestecky, 1987). The production of sIgA within the oral or nasal cavity, induced by an oral vaccine could potentially play a role in preventing the colonization of pathogens in the respiratory tract (Biesbrock et al., 1991). Buddle et al. (2008) orally vaccinated mice with Bacillus Calmette-Guerin (a vaccine against tuberculosis) and observed IgA responses in both sera and intestinal secretions, providing evidence that oral vaccines may stimulate strong cellular and humoral immunity. Similarly, Madic et al. (1995) observed marked increases in sera sIgA concentrations following intranasal inoculation of BHV-1 virus.

If oral administration of a modified-live virus (MLV) vaccine is proven to be safe and efficacious, the potential exists for commercial development of a new vaccine product that would compete with existing mucosal vaccines on the market. Presently, commercial mucosal vaccines are designed and labeled for intranasal administration only and do not contain bovine viral diarrhea virus (BVDV) antigens because the safety of an intranasal vaccine with BVDV is thought to be compromised. Oral administration of a pentavalent (bovine herpesvirus-1 (BHV-1), BVDV type 1 and 2, bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI3V) MLV vaccine may offer benefits over current intranasal mucosal vaccine products.

Furthermore, the oral route of vaccine administration may be less likely to induce clinical or subclinical effects from MLV antigens contained in the vaccine, thus making it safer and facilitating a mucosal vaccine product containing BVDV antigens. Future exploration of oral vaccine delivery via feed or water may be warranted because it would drastically reduce labor and reliance on animal handling facilities that are required for existing vaccination protocol. Oral administration of other vaccine antigens (i.e., rota-coronavirus vaccine in newborn calves, *B. abortus* in bison and wild ruminants, and rabiesvirus in various wildlife) has been shown to safely stimulate immunity in specific MALTs such as the tonsils, pharyngeal and bronchial tissue, and Peyer's patches in the small intestine (Biesbrock et al., 1991).

We hypothesized that oral administration of a pentavalent [BHV-1, BVDV type 1 and 2, BRSV, PI3V] MLV respiratory vaccine (Pyramid® 5, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) would safely and effectively stimulate local immunity, thus providing opportunity to develop a new vaccine that could enhance immunity in

cattle and improve prevention of bovine respiratory disease (BRD). Our hypothesis was tested by randomly assigning steers to 1 of 5 vaccine regimens and evaluating clinical and immunological responses of the previously unvaccinated beef calves during an 84-d observation period (Table 2.1). To our knowledge, oral administration of a MLV respiratory vaccine in cattle has not been studied previously.

Materials and Methods

General

Animal methods and procedures were approved by the IACUC committee at West Texas A&M University (WTAMU). Pyramid 5, sterile water and saline used in the study were acquired from Hereford Veterinary Supply (Hereford, TX). Appropriate vaccine handling and administrative procedures were followed using aseptic technique. A total of 42 (n = 10, each oral vaccine treatment; n = 6, positive and negative control treatments) previously unvaccinated, early-weaned beef steer calves (estimated average 90 d of age) were acquired from a single beef cow herd located in Northeast New Mexico and transported to WTAMU to begin the acclimation period. The calves arrived on 17 May 2013, were preconditioned for 30 d, and the study began (d 0) on 17 June 2013.

Blood was collected and serum analyzed using the virus neutralization (VN) assay at the Texas A&M Veterinary Medical Diagnostic Laboratory in Amarillo (TVMDL) before initiation of the study (at branding on the origin ranch; 23 March 2013; d -87) and calves were stratified according to BVDV type 1a antibody titer before treatment randomization to ensure equivalent initial BVDV titer concentrations across treatment groups. Vaccine treatments were administered to the appropriate animals (experimental unit) on d 0 and assigned to isolated pens according to treatment (10 or 6 calves/pen) at

the Ruminant Health and Immunology Research Unit at WTAMU. Treatments consisted of: 1) 1 × dose oral – pentavalent MLV respiratory vaccine (OV1; 2 mL Pyramid® 5 without Metastim administered in the buccal cavity), 2) 5 × dose oral – pentavalent MLV respiratory vaccine (OV5; 10 mL Pyramid® 5 without Metastim administered in the buccal cavity), 3) 10 × dose oral – pentavalent MLV respiratory vaccine (OV10; 20 mL Pyramid® 5 without Metastim administered in the buccal cavity), 4) positive control (PCON; 2 mL Pyramid® 5 with Metastim administered s.c., in the neck), and 5) negative control (NCON; 2 mL sterile saline administered in the buccal cavity). All calves were administered 2 mL Pyramid 5 subcutaneously in the neck on d 56 to evaluate potential anamnestic response to previous vaccine treatment.

The housing conditions were such that a 3 m buffer zone existed between pens to minimize the probability of oral-oral or aerosol contact between vaccine groups. In addition to this biosecurity measure, NCON calves were sampled first, followed by vaccinated groups to decrease the potential for virus transmission between vaccinates and controls. Throughout the acclimation and study periods, calves were allowed *ad libitum* access to water and sorghum × sudan hay along with a supplemental ration to meet nutritional requirements.

Sample collection and assay procedures

Blood samples were collected via jugular venipuncture from experimental animals at approximately 0800 beginning on d 0. Blood collected into plain evacuated tubes (Vacutainer®, SST, 10 mL tube, Ref # 367985; BD, Franklin Lakes, NJ) was allowed to clot for at least 30 min prior to centrifugation at 1,250 × g for 20 min at 20°C to obtain serum, which was allocated into triplicate aliquots and stored frozen at -20°C

until further analysis. Serum was analyzed for BVDV type 1a (Singer strain) and BRSV antibody (IgG) titer concentration on d 0, 7, 14, 28, 56, 70, and 84. An additional blood sample was collected via jugular venipuncture into evacuated EDTA-containing tubes (Vacutainer®, 7.2 mg K2-EDTA, 4 mL tube, Ref # 367861) and analyzed within 4 hr using an automated hemacytometer (ProCyte Dx Hematology analyzer; IDEXX Laboratories, Inc., Westbrook, ME) to determine total and differential peripheral blood leukocyte concentrations on d 0, 2, 4, 7, 10, 14, and 28. A daily clinical illness score (CIS) was recorded using a 0 to 4 scale by a trained investigator at approximately 0800 each morning.

Nasal and oral specimens were collected by rotating 3 nylon-flocked swabs (Puritan Medical Products #3306-PN, Guilford, ME) in the mid-naris or buccal cavity, respectively, until the swabs were completely saturated. Triplicate nasal and oral swabs were clipped, placed into sealed tubes, and stored at -20ºC until processing. Swabs were thawed at room temperature (22°C), then 300 µL phosphate buffered saline containing 1mM EDTA and 400 µL prepared Sputolysin Reagent (EMDMILLIPORE chemicals product # 560000-10ML, Billerica, MA) were added to each sample, vortexed, and incubated at room temperature for 15 min. Supernatant was harvested and analyzed for BRSV-specific IgA concentration using a commercial BRSV-specific antibody ELISA kit (Boehringer Ingelheim Svanova, Uppsala, Sweden) in which 2 µL sheep anti-bovine IgA horse radish peroxidase (HRP) conjugate (Bethyl Laboratories, Montgomery, TX) was added to the HRP conjugate provided in the kit.

The optical density (OD) values in wells coated with BRSV antigen were corrected by subtracting the OD values of the corresponding wells containing control

antigen to determine corrected OD. The mean corrected OD was then determined for all control and unknown samples for each plate. Percent positivity index (PPI) was determined as follows: PPI = corrected OD of sample or control divided by the corrected OD of positive control × 100. The mean PPI for each vaccine group on a given d was determined and reported. A sample was considered positive for BRSV-specific sIgA if the PPI index ≥ 10.

Supernatant harvested from nasal swab samples, from each treatment, was pooled within treatment and analyzed using reverse transcriptase polymerase chain reaction (RT-PCR) for detection of BVDV, PI3V, BRSV, and IBRV at TVMDL. Bovine PI3V primers were developed internally by TVMDL. Eight complete genome (all available, query date of 27 Mar 12) were MUSCLE aligned for PI-3 signatures. Selected signatures were blasted using non-redundant (nr) database to confirm specificity (max target is 100).

Statistical analyses

Dependent variables were analyzed using the PROC MIXED procedure of SAS (SAS Inst., Cary, NC) with repeated measures in a completely randomized design with animal as the experimental unit. The repeated statement was d, and fixed effects of treatment, d, and their interaction were evaluated, with pen as a random effect. Serum antibody titer data were $\log_2$ transformed prior to statistical analysis. Other variables were evaluated for normal distribution using PROC UNIVARIATE and $\log_2$-transformed prior to statistical analysis if normality was achieved. The Kenward-Roger degree of freedom method was employed to correct for unbalanced design. When a significant ($P \leq 0.05$) overall F-test existed, differences of least squares means were analyzed using the least significant difference (LSD) method (pdiff option in SAS). Contrasts were developed to

compare the mean of the 3 oral vaccine treatments to the mean of the positive or negative control group. A significance level of $\alpha = 0.05$ was used for all treatment mean or contrast comparisons.

Results and Discussion

No differences were observed for either of the contrast comparisons (oral vaccine treatments vs. PCON or NCON) for any of the variables measured in the current study. Therefore, differences reported herein reflect individual treatment mean comparison if a treatment × d interaction was significant.

Virus detection from pooled nasal swabs

Much like animals with viral infection from wild-type viruses shed and transmit virus to cohorts (CDC, 2014), animals may also shed and transmit vaccine-strain attenuated virus (Vignuzzi et al., 2008) through nasal and oral secretions (Ali et al., 2004). For this reason, we analyzed for virus presence in nasal secretions to determine if oral vaccination may have induced analogous viral shedding. Results indicate BRSV was present in the herd on and presumably prior to d 0 (Table 2.2). Unfortunately, because the calves were likely exposed to wild-type BRSV prior to vaccine treatment administration on d 0, BRSV-specific response variables measured in this study were likely confounded. This observation provides clarification as to why the BRSV-specific sIgA results were erratic, and explains why serum BRSV-specific IgG titers were increased on d 28 across all treatments, including the negative control group.

Interestingly, all pooled nasal swab samples were PI3V negative on d 0; however, PI3V was detected on d 7 and 14 in the oral vaccinates only, and the prevalence and concentration (based on CT values) of PI3V appeared to be dose dependent among the

oral vaccine treatments (Table 2.3). This would suggest that the higher doses of oral vaccine administered resulted in vaccine-induced shedding of PI3V because samples were negative prior to vaccination. Prevalence of wild type PI3V in the field is typically low, and all samples from the control treatments were negative throughout. However, further genotype analysis of the PI3V isolate is required to confirm this interpretation. No BHV-1 was detected from nasal swab samples in this study, and only one of the pooled samples, on d 28, was observed to be BVDV positive (Table 2.4).

Serum BRSV- and BVDV-specific antibody titers

Results of BRSV- and BVDV-specific serum antibody titers are illustrated in Figures 2.1 and 2.2, respectively. An effect of d on BRSV-specific antibody titer was observed ($P < 0.001$); notably, BRSV antibody titer concentration increased for all treatments between d 14 and 28. The increase in circulating BRSV-specific antibody would be expected for vaccinated groups during this time. However, a similar increase observed for the NCON treatment was not expected, yet this observation is explained by the likely presence of wild type BRSV in the herd on d 0, as previously discussed.

Serum BVDV-specific antibody titer concentration is presented in Figure 2.2. The oral vaccine treatments and NCON did not display increased BVDV antibody titer until subsequent parenteral vaccination on d 56. However, PCON was greater than OV1 on d 56 ($P = 0.017$) and 70 ($P = 0.021$). This observation suggests that oral vaccine administration did not clearly stimulate BVDV-specific antibody production. Further, only one pooled nasal swab sample tested BVDV positive via RT-PCR (Table 2.4), demonstrating that viral shedding of BVDV did not occur following oral vaccination.

Following parenteral vaccination on d 56, BVDV-specific antibody titer increased overall (d effect; $P < 0.001$).

Complete blood count

A treatment × d interaction was observed for total peripheral blood leukocyte (PBL) concentration ($P = 0.001$; Figure 2.3). Neutrophils were greatest ($P \leq 0.05$) for PCON on d 4 (Figure 2.4). On d 14, eosinophils were greater for PCON compared to the oral vaccine treatments ($P \leq 0.05$), yet did not differ from NCON ($P = 0.19$; Figure 2.5).

An interesting increase in basophils, which are known for their role in allergic reaction, was observed for OV10 on d 2 ($P < 0.001$); this may suggest an allergenic response to the highest oral vaccine dose occurred (Figure 2.6).

Furthermore, PBL decreased overall as the study progressed (d effect; $P < 0.001$). Red blood cell count ($P < 0.001$), hemoglobin ($P < 0.001$) and hematocrit ($P < 0.001$) decreased with time; whereas, an overall increase in platelets was observed ($P < 0.001$). These changes in hematological variables over time may suggest acute infection, physiological stress, and/or inflammation during the study period.

BRSV-specific secretory IgA

No differences ($P = 0.68$) in the PPI of sIgA from oral swab samples were observed among treatments. The OV5 group had an increased PPI for nasal sIgA on d 0. This observation was not expected, since cattle had not been vaccinated against BRSV prior to study initiation, and prompted the RT-PCR analysis for the presence of BRSV in the nasal swab samples.

Multiple studies have demonstrated efficacy of oral vaccination in cattle with other antigens. Buddle et al. (2005) orally administered *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) in a lipid-based formulation prior to challenging cattle with virulent *M. bovis.* Positive control animals were vaccinated subcutaneously with BCG and negative controls were not vaccinated. Following the challenge, orally vaccinated cattle demonstrated significant protection against bovine tuberculosis compared to negative controls, and were comparable to immunity stimulated in subcutaneously vaccinated animals. Elzer et al. (1998) orally vaccinated sexually mature heifers against *Brucella abortus* prior to *B. abortus* challenge. Heifers were orally vaccinated by mixing viable organisms with feed. Six weeks following vaccination via feed, heifers were bred to brucellosis-free bulls, and then challenged orally with *B. arbortus,* 186 d into gestation. Following challenge and parturition, the bacterial strain 2308 was recovered from 80% of controls but only 20% of vaccinates. Additionally, 30% of the vaccinates delivered dead, premature, or weak calves, whereas 70% of controls had dead or weak calves, demonstrating efficiency of the oral vaccine.

Oral vaccination of calves against salmonellosis, using *Salmonella typhimurium* strain induced protection in seven-d-old Fresian calves. Eight of the ten calves used in the experiment were challenged with oral *Salmonella* strains after vaccination and seven of the eight survived. Four unvaccinated control animals were challenged and all scoured severely and were euthanized, demonstrating that experimental challenge with *S. typhimurium* is protected against using oral vaccination with live-attenuated strains of the bacterium (Jones et al., 1991).

Most studies involving oral vaccination in cattle have used bacterial, rather than viral, antigens. The reason for a lack of exploration of viral antigens is unclear, yet it could potentially be due to the difference in replication between viruses and bacteria. Bacteria are able to replicate on their own, whereas viruses must have a viable host cell in which to replicate (Fisher, 2014). Viruses inject their genetic material into the cells of animals to achieve replication and are continually mutating to create diversity, in order to evade the immune system (Fleischmann, 1996). Perhaps this molecular occurrence makes effective oral vaccination with live-attenuated viruses more difficult to achieve when vaccines do not contain large quantities of viral antigen or antigens are not delivered alongside an adjuvant or protective delivery mechanism.

Literature observing oral administration of live-attenuated viruses in cattle is limited, and no previous studies evaluating MLV respiratory antigens were found. De Leeuw et al. (1980) administered a commercially available oral vaccine against live-attenuated rotavirus in dairy calves and observed no differences in the incidence rate or severity of undifferentiated neonatal calf diarrhea between unvaccinated control calves (n=76) and vaccinates (n=74). A challenge phase was not included in this study, so it is unclear if the natural pathogen load was adequate to detect differences. Conversely, Khandelwal et al. (2003) orally vaccinated cattle against rinderpest virus (RPV), using transgenic peanut plants expressing hemagglutinin protein of RPV, and observed hemagglutinin-specific serum antibody production in the cattle. This study did not involve the use of an adjuvant. However, the results in our study suggest that an adjuvant or protective delivery mechanism, that is immunostimulatory to mucosal tissues, may be

necessary to stimulate mucosal and/or systemic immunity in calves when vaccinating against respiratory viruses in cattle.

Bowersock et al. (1998) used oral administration of a model antigen (ovalbumin), protected in alginate microspheres, to mimic pulmonary immunity in cattle. Calves were vaccinated orally with ovalbumin following initial "priming" doses of either subcutaneously- or orally-administered ovalbumin. Calves receiving both the oral priming dose and oral booster demonstrated induction of pulmonary immunity evidenced by increased numbers of antigen-specific IgA in bronchoalveolar lavage fluids. Calves receiving the subcutaneous priming dose and oral booster elicited a greater number of antigen-specific IgA and also had increased numbers of antigen-specific antibodies in the peripheral blood, whereas the oral/oral calves did not. Nevertheless, this study demonstrated the ability of antigen encapsulated in alginate microspheres to stimulate mucosal immunity in calves. In mice, oral immunization with reovirus encapsulate in alginate microspheres induced mucosal immunity (intestinal sIgA), while unencapsulated antigen did not, suggesting that breakdown of viral antigen in the digestive tract may be a challenge to oral vaccination against viruses (Periwal et al., 1997).

Conclusion

There is limited evidence of a detectable immune response to oral administration of a pentavalent MLV respiratory vaccine in this study; however, the presence of post-vaccination PI3V shedding, in oral vaccinates only, warrants attention. Real time PCR analysis of nasal swabs indicated shedding of BRSV on d 0 and throughout the study, across all treatments, which suggests the cattle used in this experiment were exposed to wild-type BRSV prior to study initiation. Unfortunately, this would confound the BRSV-

specific response variables. Many challenges exist regarding oral vaccination of ruminants (Shewen et al., 2009). If MLV antigen encounter does not occur in the tonsilar crypts or lymph nodes upon initial passage, the survivability of unprotected (unencapsulated) MLV antigens in the rumen environment is likely short-lived and therefore secondary antigen capture in the gastrointestinal tract is not plausible (Bowersock et al., 1999).

Other differences provide indirect evidence of efficacy of the oral route of MLV vaccine administration. Basophils were increased by d 2 for the OV10 treatment, PCON had greater BRSV antibody titer compared to NCON, while oral vaccine treatments were intermediate, and nasal BRSV-specific sIgA increased, albeit slightly, post-vaccination. Further research is warranted to investigate the potential for oral administration of MLV respiratory vaccine in cattle, testing alternative delivery systems that protect orally-delivered antigen, such as particulate delivery systems or live vectors.

Table 2.1. Description of vaccine treatment regimens.

Treatment 1 (n = 10)	Treatment 2 (n = 10)	Treatment 3 (n = 10)	Treatment 4 (n = 6)	Treatment 5 (n = 6)
1 x dose oral vaccination (OV 1)	**5 x dose oral vaccination (OV5)**	**10 x dose oral vaccination (OV 10)**	**Positive Control (PC)**	**Negative Control (NC)**
2 mL Pyramid® 5 vaccine without Metastim administered in the buccal cavity	10 mL Pyramid® 5 vaccine without Metastim administered in the buccal cavity	20 mL Pyramid® 5 vaccine without Metastim administered in the buccal cavity	2 mL Pyramid® 5 vaccine with Metastim administered parenteral (s.c.) in the neck	2 mL sterile saline administered in the buccal cavity

Table 2.2. Real time PCR results for the detection of BRSV in pooled nasal swab samples.

Day	NCON[a]	PCON	OV1	OV5	OV10
0	+[b]	-	-/++[c, d]	-/+	+/+
7	+	+	+/++	+/+	+/++
14	+	-	-/+	-/+	-/-
28	-	-	-/-	-/-	-/-

[a]NCON=negative control; PCON=positive control; OV1=1x oral dose Pyramid 5; OV5=5x oral dose Pyramid 5; OV10=10x oral dose Pyramid 5

[b]Plus/minus signs indicate positive/negative RT-PCR detection of BRSV within pooled samples.

[c]Two separate pools were analyzed from OV1, OV5, and OV10. Results from each pool are represented within one box in the table, separated by a forward slash.

[d]++ indicates a strong positive result, based on a CT value < 27.

Table 2.3. Real time PCR results for the detection of PI3V in pooled nasal swab samples.

Day	NCON[a]	PCON	OV1	OV5	OV10
0	-[b]	-	-/-[c]	-/-	-/-
7	-	-	-/-	-/+	+/++[d]
14	-	-	+/-	-/+	-/+
28	-	-	-/-	-/-	-/-

[a] NCON=negative control; PCON=positive control; OV1=1x oral dose Pyramid 5; OV5=5x oral dose Pyramid 5; OV10=10x oral dose Pyramid 5

[b] Plus/minus signs indicate positive/negative PCR detection of BRSV within pooled samples

[c]Two separate pools were analyzed from treatments OV1, OV5, and OV10. Results from each pool are represented within one box in the table, separated by a forward slash.

[d]++ indicates a strong positive result, based on a CT value < 27.

Table 2.4. Real time PCR results for the detection of BVDV in pooled nasal swab samples.

Day	NCON[a]	PCON	OV1	OV5	OV10
0	-[b]	-	-/-[c]	-/-	-/-
7	-	-	-/-	-/-	-/-
14	-	-	-/-	-/-	-/-
28	-	-	-/-	-/-	+/-

[a] NCON=negative control; PCON=positive control; OV1=1x oral dose Pyramid 5; OV5=5x oral dose Pyramid 5; OV10=10x oral dose Pyramid 5

[b]Plus/minus signs indicate positive/negative RT-PCR detection of BVDV within pooled samples.

[c] Two separate pools were analyzed from OV1, OV5, and OV10. Results from each pool are represented within one box in the table, separated by a forward slash.

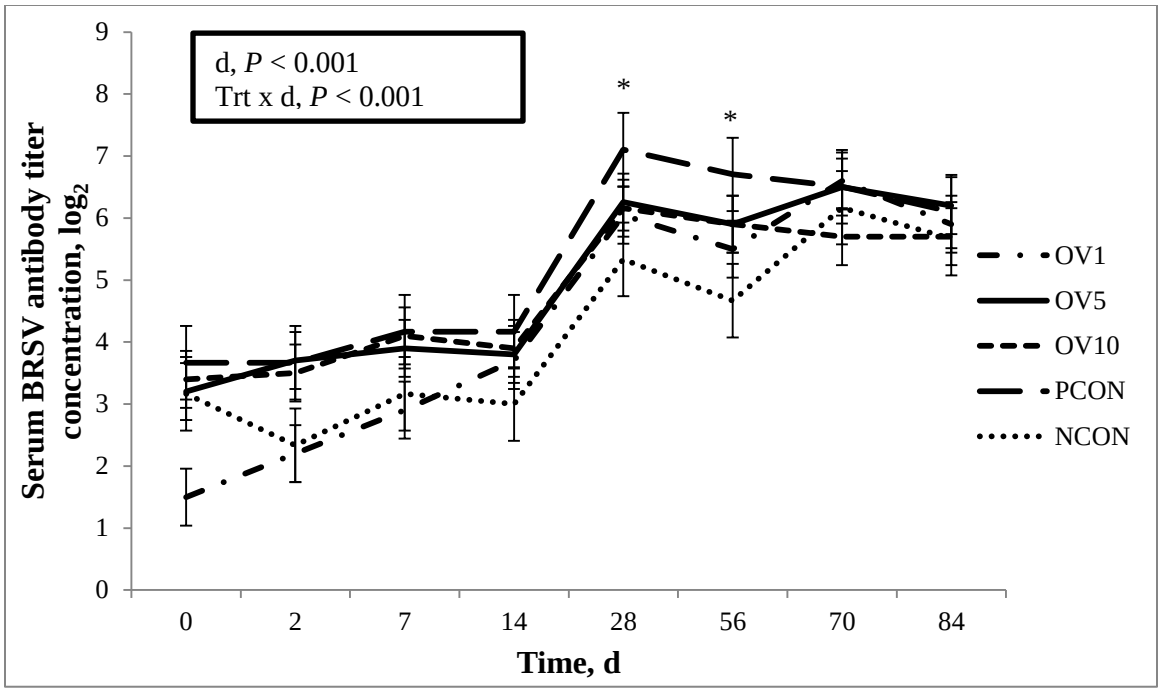

Figure 2.1. Effect of vaccine treatment on serum BRSV-specific antibody titer concentration. *PCON differs from NCON, $P \leq 0.04$. NCON=negative control; PCON=positive control; OV1=1x oral dose Pyramid 5; OV5=5x oral dose Pyramid 5; OV10=10x oral dose Pyramid 5.

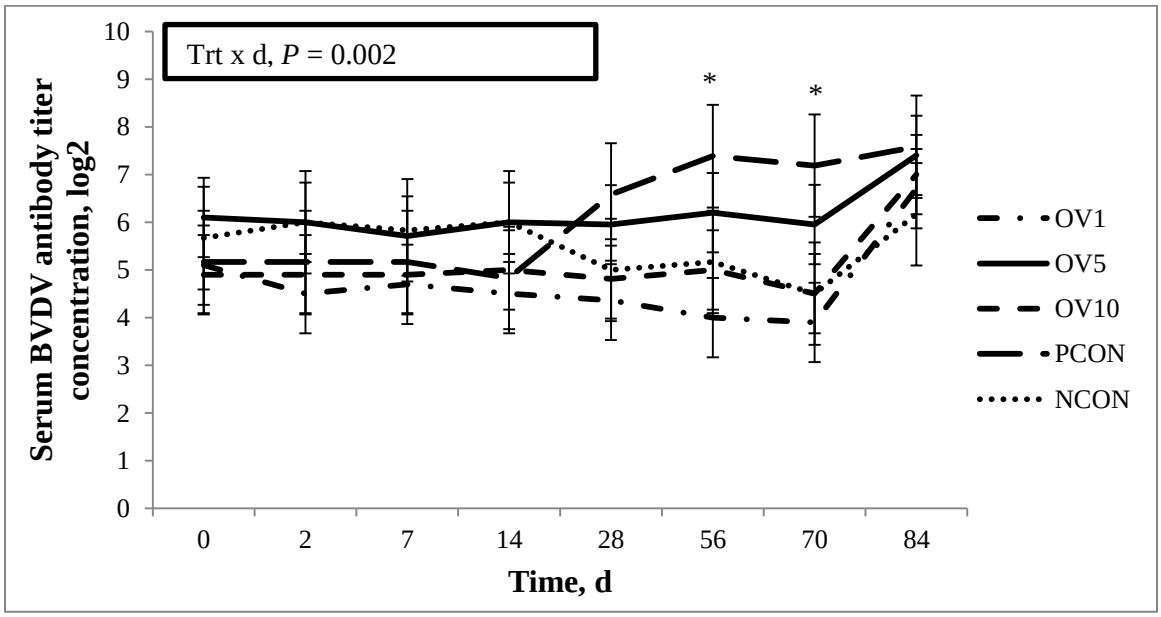

Figure 2.2. Effect of vaccine treatment on serum BVDV-specific antibody titer concentration. *PCON differs from OV1, P≤0.02. NCON=negative control; PCON=positive control; OV1=1x oral dose Pyramid 5; OV5=5x oral dose Pyramid 5; OV10=10x oral dose Pyramid 5.

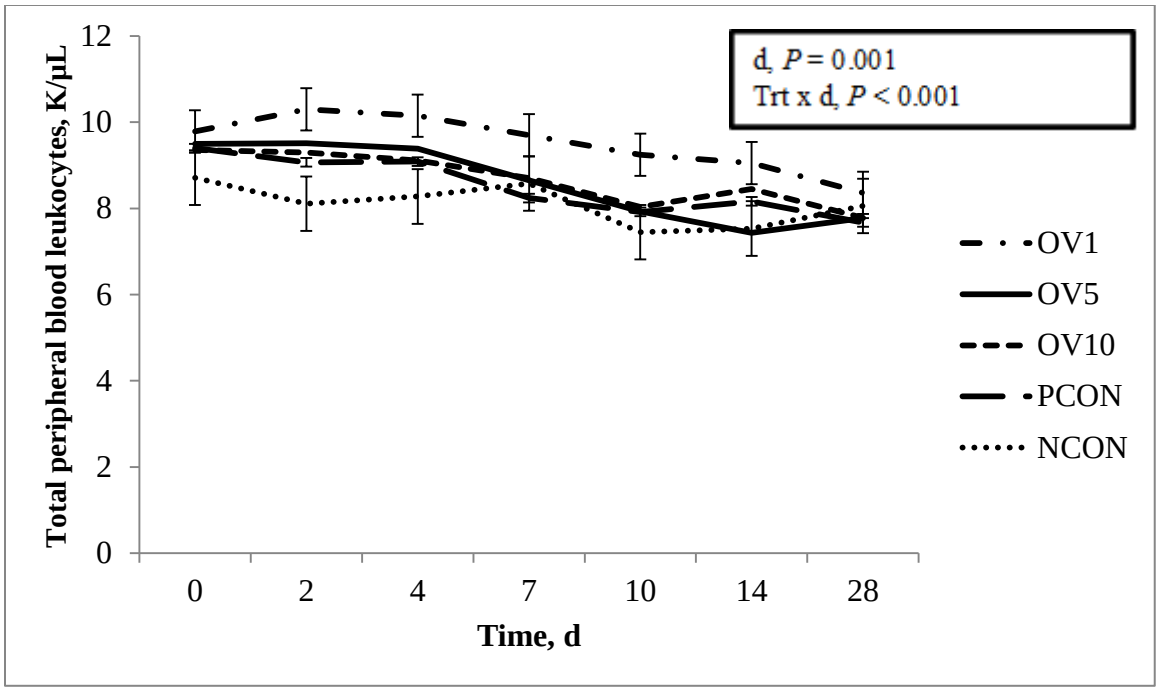

Figure 2.3. Effect of vaccine treatment on total peripheral blood leukocyte concentration. NCON=negative control; PCON=positive control; OV1=1x oral dose Pyramid 5; OV5=5x oral dose Pyramid 5; OV10=10x oral dose Pyramid 5.

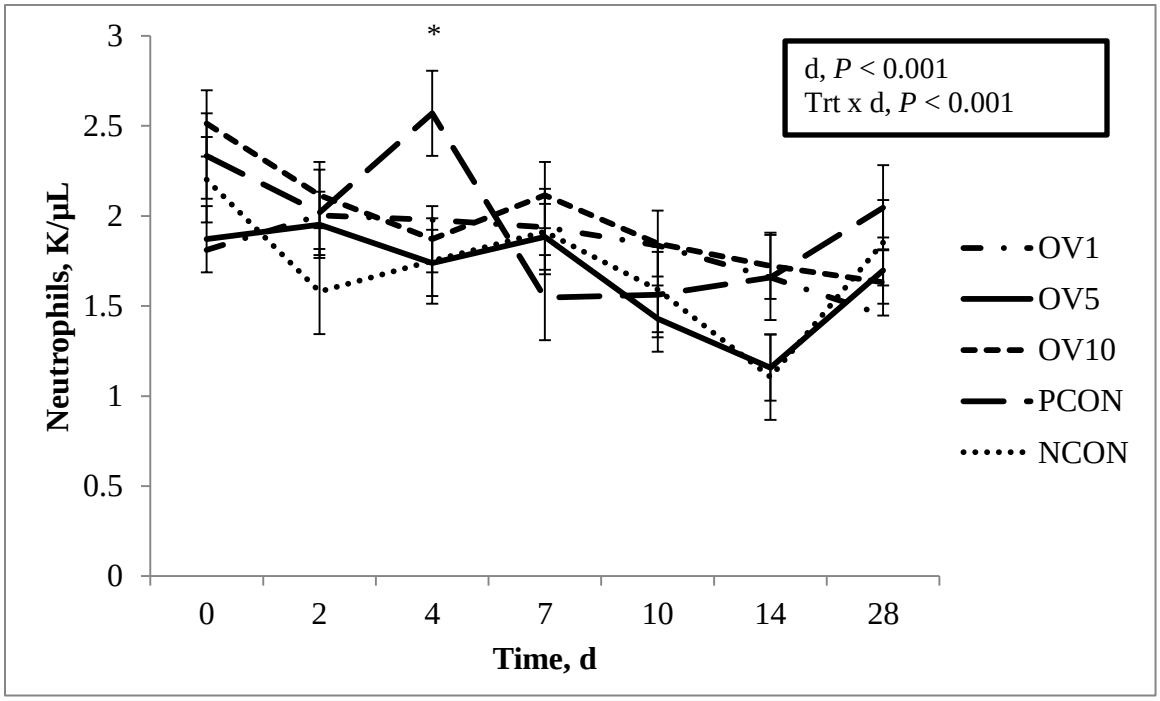

Figure 2.4. Effect of vaccine treatment on neutrophil concentration. *PCON differs from all other treatments, P≤0.05. NCON=negative control; PCON=positive control; OV1=1x oral dose Pyramid 5; OV5=5x oral dose Pyramid 5; OV10=10x oral dose Pyramid 5.

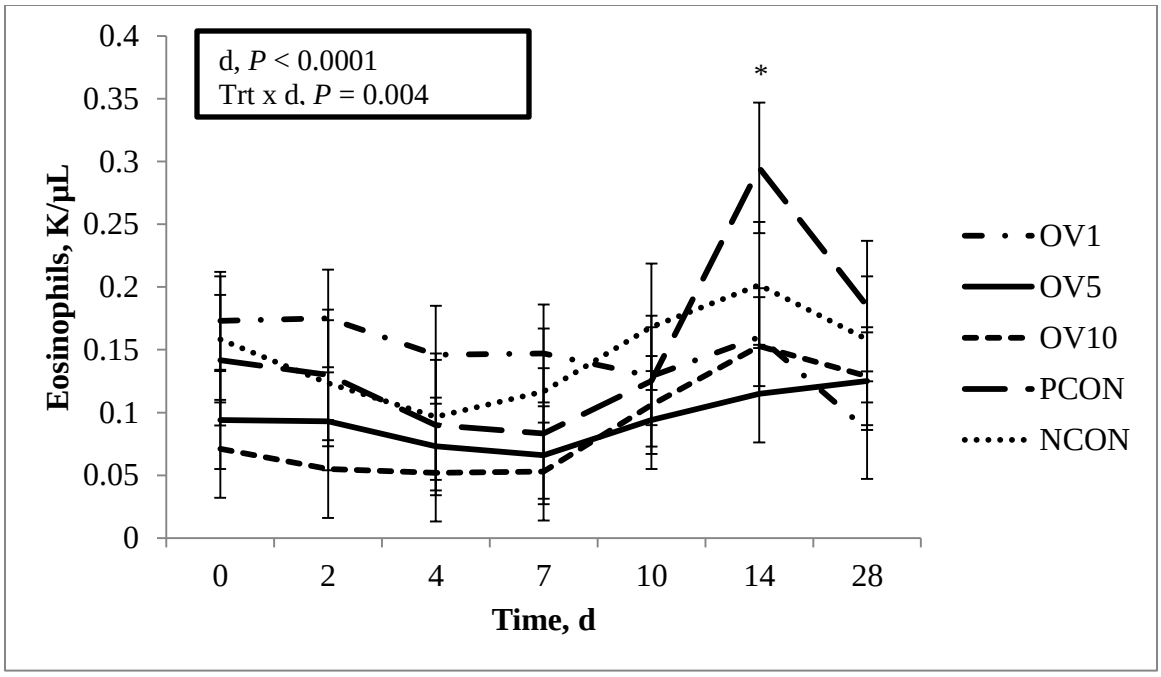

Figure 2.5. Effect of vaccine treatment on eosinophil concentration. *PCON differs from all oral vaccine treatments, $P \leq 0.04$. PCON=positive control; OV1=1x oral dose Pyramid 5; OV5=5x oral dose Pyramid 5; OV10=10x oral dose Pyramid 5.

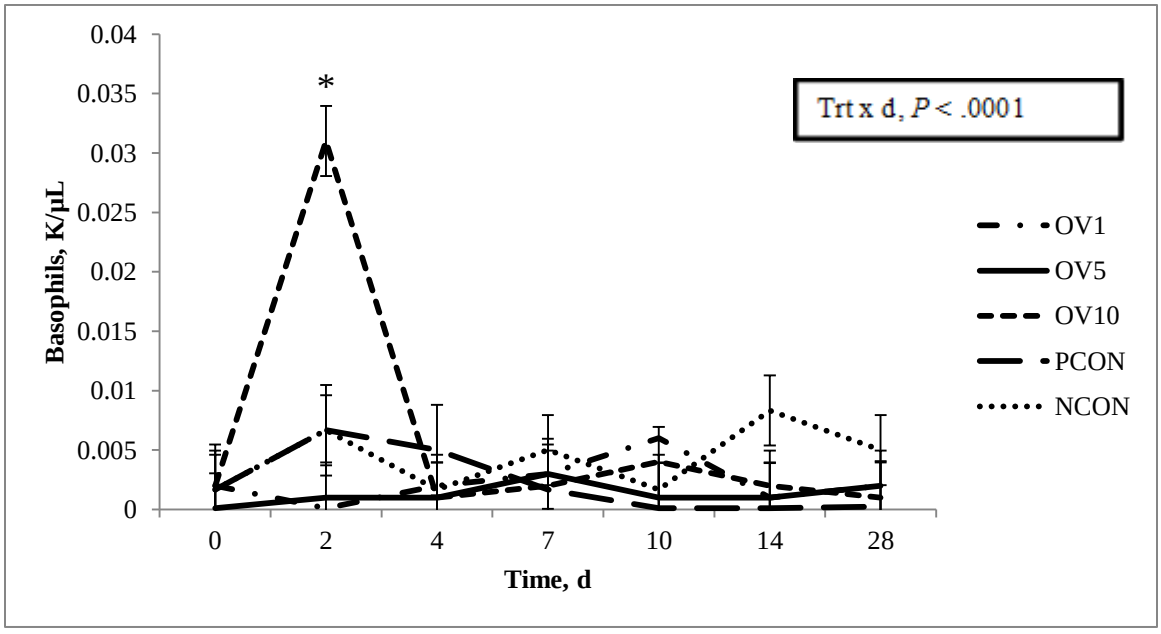

Figure 2.6. Effect of vaccine treatment on basophil concentration. *OV10 differs from all other treatments, $P < 0.001$. PCON=positive control; OV1=1x oral dose Pyramid 5; OV5=5x oral dose Pyramid 5; OV10=10x oral dose Pyramid 5.

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CHAPTER III

INTRANASAL ADMINISTRATION OF A PENTAVALENT RESPIRATORY VACCINE CONTAINING WATER-IN-OIL ADJUVANT DOES NOT STIMULATE MUCOSAL OR SYSTEMIC IMMUNITY IN BEEF CALVES

H. D. Hughes

West Texas A&M University

ABSTRACT

Intranasal administration of modified-live virus (MLV) respiratory vaccines in cattle may be beneficial at stimulating mucosal immunity of the nasopharyngeal tissues and prevent respiratory virus pathogens from infecting and replicating within host cells. A total of 30 clinically healthy, crossbred beef heifer calves were stratified by pre-trial serum BVDV type 1a titer concentration, then assigned randomly to 1 of 3 vaccine treatment regimens (n = 10) consisting of: 1) intranasal administration of saline (1mL/naris) on d 0 and parenteral vaccination with Pyramid 5 on d 29 (CON-PYR), 2) intranasal administration of Inforce 3 (1 mL/naris) on d 0 and parenteral vaccination with Bovi-shield Gold 5 on d 29 (INF-BSG), and 3) intranasal administration of Prism 5 with Metastim (1mL/naris) on d 0 and parenteral vaccination with Pyramid 5 on d 29 (PRM-PYR). Calves (experimental unit) within vaccine treatments were housed in 1 of 2 pens separated by an empty pen and contained 5 animals each (6 pens total). The INF-BSG group exhibited greater ($P \leq 0.06$) serum BRSV-specific antibody titer than CON-PYR or PRM-PYR on d 14, 29 and 42. Similarly, BRSV-specific secretory IgA from nasal secretions was detected for INF-BSG on d 7, 10, 14, 28 and 42 but not for CON-PYR or PRM-PYR. There was no serum BVDV type 1a antibodies detected prior to administering parenteral vaccines on d 29. However, cattle vaccinated with PYR on d 29 (CON-PYR and PRM-PRY treatments) exhibited greater ($P < 0.001$) serum BVDV type 1a antibody titer on d 42, 56 and 84, which suggests that the BVDV-specific vaccine response was improved for PYR vs. BSG. No differences ($P > 0.05$)

in rectal temperature or clinical illness score were observed, and IFN-α and BVDV-specific secretory IgA were not detected from nasal swab supernatant in this experiment. Results of the current study suggest that Prism 5 is not a clearly effective intranasal vaccine; yet, further research is needed to determine the efficacy of a squalane-based (water-in-oil) adjuvant (i.e., Metastim) for immune enhancement of vaccine antigens delivered in the respiratory mucosa of beef calves.

Introduction

Mucosal surfaces, including the interior of the nose and mouth, are the primary location that viral and bacterial pathogens enter a host, and the mucosa-associated lymphoid tissue (MALT) of the respiratory tract is a critical mechanism for respiratory pathogen entry and immune defense. Currently, a limited number of intranasal vaccines exist for prevention of bovine respiratory disease (BRD); however, there is not a commercial vaccine labeled for intranasal administration that contains bovine viral diarrhea virus (BVDV) antigens, due to safety concerns associated with potential immunosuppression from BVDV antigen inclusion.

Intranasal vaccines currently consist of modified-live viruses (MLV), including bovine herpesvirus-1 (BHV-1), bovine respiratory syncytial virus (BRSV), and parainfluenza-3 virus (PI-3V); primary viral pathogens implicated in BRD cases. One such intranasal vaccine is marketed under the trade name Inforce 3 (Zoetis, Kalamazoo, MI). Since Inforce 3 (or other intranasal vaccine products) contains no BVDV antigen, intranasal administration of a pentavalent respiratory vaccine [BVDV type 1 and 2 (killed strains), BHV-1, BRSV, PI-3V (live-attenuated strains)], currently labeled for parenteral administration, may offer benefits over existing intranasal (mucosal) vaccine products.

The primary objective of the current study was to evaluate whether intranasal administration of a multivalent respiratory vaccine marketed under the trade name Prism 5 (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO), would safely and effectively stimulate local and/or systemic immunity, thus providing opportunity to develop a new intranasal vaccine product that may enhance immunity in beef and dairy cattle and improve prevention of BRD.

Materials and Methods

General

Animal methods and procedures were approved by the IACUC committee at West Texas A&M University (WTAMU). Vaccines used in this study were acquired from Hereford Veterinary Supply (Hereford, TX). Appropriate vaccine handling procedures were closely followed and vaccines were administered using aseptic technique.

A total of 30 (n = 10; 6 isolated pens containing 5 animals/treatment) clinically healthy, crossbred beef heifer calves were stratified by pre-trial serum BVDV type 1a antibody titer, then assigned randomly to vaccine treatments (Table 4.1). Treatments consisted of 1 of 3 vaccine treatment regimens (n = 10 calves/treatment) consisting of: 1) intranasal administration of saline (1mL/naris) on d 0 and parenteral vaccination with Pyramid 5 on d 29 (CON-PYR), 2) intranasal administration of Inforce 3 (1 mL/naris) on d 0 and parenteral vaccination with Bovi-shield Gold 5 on d 29 (INF-BSG), and 3) intranasal administration of Prism 5 with Metastim water-in-oil adjuvant (1mL/naris) on d 0 and parenteral vaccination with Pyramid 5 on d 29 (PRM-PYR). Cattle were administered a parenteral respiratory vaccine on d 29 to assess potential anamnestic response from previous intranasal antigen administration; CON-PYR and PRM-PYR treatments received 2 mL of Pyramid 5, subcutaneously (SC) and the INF-BSG treatment received 2 mL Bovi-Shield Gold 5, SC.

Treatment administration and sample collection

Blood was collected and serum was harvested and analyzed using the virus neutralization assay at the Texas A&M Veterinary Medical Diagnostic Laboratory –

Amarillo, TX (TVMDL) prior to study initiation to facilitate stratification by serum BVDV type 1a antibody titer.

Vaccine treatments were assigned randomly and administered to the appropriate animals on d 0 and animals were allocated to isolated treatment pens at the WTAMU Research Feedlot. Calves (experimental unit) within vaccine treatments were housed in 1 of 2 pens containing 5 animals each (6 pens total). Cattle in the CON treatment pens were handled first at each collection time to facilitate biosecurity. Calves were allowed *ad libitum* access to water and a common growing ration during the 84-d study. Blood samples were collected via jugular venipuncture from animals at approximately 0700 beginning on d 0 (17 Mar 2014), and on the appropriate sampling d through d 84 (09 Jun 2014).

Blood was collected into plain evacuated tubes (Vacutainer®, Becton Dickinson, Franklin Lakes, NJ; 10 mL tube, Ref # 367985), allowed to clot for at least 30 min, and serum was decanted into triplicate aliquots following centrifugation at 1,250 × *g* for 20 min at 20° C. Serum BRSV and BVDV type 1a antibody titer concentrations were evaluated from d 0, 14, 29, 42, 56, and 84.

An additional blood sample was collected via jugular venipuncture into evacuated EDTA tubes (Vacutainer®, 7.2 mg K2-EDTA, 4 mL tube, Ref # 367861) , and analyzed within 4 hrs at TVMDL to determine total and differential peripheral blood leukocyte concentrations on d 0, 2, 4, 7, 10, 14, and 29. Rectal temperature was also recorded on d 0, 2, 4, 7, 10, 14, and 29. For the duration of the study, daily clinical illness score (CIS) was observed for each animal using a 0 to 4 scale by a trained investigator at approximately 0800.

Nasal swab specimens were collected by rotating 3 nylon-flocked swabs (Puritan Medical Products #3306-PN, Guilford, ME) in the mid-naris (prior to vaccine treatment administration, on d 0), until the swabs were completely saturated. The triplicate nasal swabs were clipped, placed into sealed tubes, and stored at -20ºC until time of processing. Swabs were thawed at room temperature, then 300 μL phosphate buffered saline containing 1mM EDTA and 400 μL prepared Sputolysin Reagent (EMDMILLIPORE chemicals product # 560000-10ML, Billeria, MA) were added to each sample, and samples were vortexed and incubated atroom temperature for 15 min. Supernatant was harvested and analyzed to quantify BRSV-specific and BVDV-specific sIgA (d 0, 2, 4, 7, 10, 14, 29, and 42), and IFN-α concentration (d 0, 2, 4, and 7) using commercial ELISA kits (Boehringer Ingelheim Svanova, Uppsala, Sweden and Kamiya Biomedical Company, Seattle WA, respectively).

For BRSV- and BVDV-specific sIgA, the optical density (OD) values in wells coated with BRSV or BVDV antigen were corrected by subtracting the OD values of the corresponding wells containing control antigen to determine corrected OD. The mean corrected OD was then determined for all control and unknown samples for each plate. Percent positivity index (PPI) was determined as follows: PPI = corrected OD of sample or control/corrected OD of positive control × 100. The mean PPI for each vaccine group on a given d was determined. A sample was considered positive for BRSV- or BVDV-specific sIgA if the PPI $\geq$ 10.

Interferon (IFN)-α concentration in nasal swabs was evaluated using a standard curve, derived from serial dilution of bovine IFN-α calibrator provided in the ELISA kit. Sample duplicate readings were averaged and their values subtracted from the average

zero calibrator OD and 4-parameter logistic regression of the standard curve was used to extrapolate IFN-α concentration of unknown samples for statistical analysis.

Statistical analyses

A completely randomized design was used with animal serving as experimental unit. Dependent variables were analyzed using the MIXED procedure of SAS (SAS Inst., Cary, NC) with repeated measures. The repeated statement was d, and effects of treatment, d, and their interaction were evaluated. The Kenward-Roger degree of freedom method was employed and the covariance structure which generated the lowest Akaike's Information Criterion (AIC) value was implemented for each dependent variable. Statistical significance was established for a resulting *P*-value ≤ 0.05 and a tendency was considered between 0.06 and 0.10.

Results and Discussion

No differences in rectal temperature ($P \geq 0.10$) were observed across d or between treatments. There was no clinical morbidity or mortality observed in this experiment. Baseline concentrations of IFN-α were different among treatments on d 0, prior to treatment administration; however, no difference ($P > 0.05$) in IFN-α present in nasal secretions was observed following intranasal vaccine administration. This observation was not expected, as intranasal administration of viral vaccine antigens (INF-BSG and PRM-PYR treatments) should stimulate leukocytes to produce IFN-α. Conversely, Woolums et al. (2004) observed an increase in IFN-γ in lymph node tissue specimens of young Holstein calves intranasally vaccinated with live-attenuated BRSV antigen, suggesting stimulation of innate immunity by intranasal vaccination.The extraction process designed for sIgA detection in nasal swab samples, which was similarly used for

IFN-α detection, may have been problematic, or issue with the commercial ELISA kit may have resulted in the lack of a detectable treatment response in the current study.

Complete blood count

No differences ($P = 0.86$) in total peripheral blood leukocyte concentrations were observed among treatments following intranasal vaccination. A treatment × d interaction was observed for eosinophil concentration. Cattle on PRM-PYR had the greatest ($P < 0.001$) eosinophil concentration on d 7; however, all concentrations were within the normal reference range for the differential leukocytes, so it is unclear if this was due to treatment or random variation. No other differences ($P \geq 0.49$) in hematological variables were observed in this study.

BRSV-specific sIgA and serum BRSV antibody titer

Figure 4.1 illustrates the percent positivity index (PPI) of BRSV-specific secretory (s) IgA isolated from nasal swabs. While there was not a significant treatment effect observed ($P = 0.12$), cattle receiving INF-BSG displayed a numerically greater PPI than cattle in the CON-PYR and PRM-PYR treatment groups. (Note: the BRSV sIgA assay is semi-quantitative, and PPI values < 10 are considered negative; whereas, values ≥ 10 are considered positive). Similarly, serum BRSV antibody titer was increased for INF-BSG (Fig. 4.2); yet, the serum BRSV antibody concentration was low for CON-PYR and PRM-PYR throughout the course of this experiment. The lack of BRSV-specific antibody responses observed in the early portion of this study for PRM-PYR may have been affected by the Metastim oil-in-water adjuvant contained in PRM, but not INF. Other studies have demonstrated increases in serum BRSV antibody titer following

intranasal vaccination with MLV BRSV vaccine (Ellis et al., 2001; Woolums et al., 2004).

A squalane-based water-in-oil adjuvant such as Metastim is designed to enhance the immune response to vaccine antigens administered parenterally, rather than in the epithelial environment of the respiratory mucosa. It is possible that the Metastim adjuvant contained in PRM-PYR is inhibitory to mucosal breakthrough via M cells, and subsequent antigen capture by dendritic cells that reside in the mucosal environment for antigen processing and presentation or the adjuvant may promote mucociliary clearance of PRM-PYR compared to IFN-BSG. Alternatively, differences in the concentration of BRSV antigen between INF-BSG and PRM-PYR may have impacted the BRSV immune responses evaluated in this study. Cattle were revaccinated on d 29, and those that received INF-BSG had a more profound increase in serum BRSV antibody ($P < 0.001$) compared to PRM-PYR.

BVDV-specific sIgA and serum BVDV type 1a antibody titer

We were unable to detect the presence of BVDV-specific sIgA for any of the treatments, post-intranasal vaccination (samples from d 0, 2, 4, 7, 10, 14, 29 or 42). Results of serum BVDV type 1a antibody titers are illustrated in Figures 4.3 (Singer strain) and 4.4 (NADL strain). Between d 0 and 29 (i.e., prior to parenteral vaccination), no BVDV antibody was detected in serum. These results were expected among the CON-PYR and INF-BSG treatments, since neither received BVDV antigen in the initial intranasal procedure. However, the absence of BVDV-specific sIgA and serum antibody titer prior to parenteral administration of PYR on d 29 suggest a lack of immunogenicity existed for the intransal PRM procedure.

Although PRM contains killed versions of BVDV type 1 and 2, it did not induce a local (i.e., sIgA) systemic BVDV antibody titer response (i.e., IgG) when administered intranasal. This is perhaps either because the killed BVDV antigen itself is not immunogenic to the degree that Th2 immunity is clearly stimulated after administration in the naris or due to effects of the Metastim water-in-oil adjuvant contained in the PRM vaccine, as previously discussed. One other study (Xue et al.,2010) has investigated the immunogenicity intranasally administering a commercially available MLV pentavalent vaccine designed for parenteral administration, containing BVDV types 1 and 2. Minimal serum antibody titers to BVDV were observed after aerosolized BVDV type 1 challenge; however, only one-fifth (3/15) of vaccinated cattle developed clinical signs compared to all of the control calves (16/16). The authors did not indicate a trade name of the vaccine used in that study.

Following parenteral vaccination on d 29 with either PYR (CON-PYR and PRM-PYR groups) or BSG (INF-BSG group), cattle receiving PYR had greater serum BVDV-specific antibody titers on d 56 and 84 (Singer strain; $P < 0.001$). We suggest the increases in serum BVDV antibody observed after parenteral vaccination on d 29 is largely due to enhanced immunogenicity resulting from Metastim water-in-oil adjuvant when administered parenterally, yet as previously discussed, this adjuvant may have had an opposing effect when administered intranasally. When sera were evaluated using NADL strain in the same virus neutralization assay, results were similar; however, the difference in magnitude between treatments was less. This observation is likely due to the fact that the NADL strain used in this VN assay is homologous to the BVDV strain contained in BSG.

Overall, the BVDV-specific outcomes suggest greater immunogenicity of PYR (CON-PYR and PRM-PYR) compared to BSG (INF-BSG), but PRM administered intranasally on d 0 (PRM-PYR), which contained the same Metastim water-in-oil adjuvant included in PYR, did not result in a clear BVDV-specific immune response.

Most vaccines administered to beef cattle in the US are parenteral, which results primarily in a systemic immune response. However, potential exists for intranasal vaccination of respiratory antigens, as a way to combat pathogens at the local point of entry; the nose and mouth. A commercial intranasal vaccine containing BVDV antigens does not currently exist, which proves to be a challenge if administering an intranasal vaccine alone because this virus can directly or indirectly attribute to respiratory and reproductive diseases affecting cattle.

In the current study, it is unclear if the reduced BRSV-specific immune responses in PRM-PYR cattle was due to a lower viral antigen concentration (TCID) compared to INF-BSG cattle and/or due to effects of the water-in-oil adjuvant (Metastim) contained in the PRM vaccine, which is designed for parenteral, rather than intranasal administration. It is possible that the Metastim adjuvant in PRM inhibits the encapsulated antigen from making efficient contact with mucosal surfaces (thus greater mucociliary clearance) or inhibits mucosal penetration after contact, since it is a squalane emulsion.

Squalane is a precursor to cholesterol found in the liver of sharks and other fishes (Allison, 1999). Advantages of this adjuvant are numerous, including long-term stability of up to 6 yrs at ambient temperature, terminal filtration which ensures sterility of the final product containing the antigen, and the ability to pass from injection sites into the lymphatics and lymph nodes of the drainage chain, blood, and spleen due to small

particle size (Allison, 1999). However, without the induction of sIgA production in mucosal tissues, this adjuvant may not be efficacious when administered intranasally. Previous research with squalane oil consisting of water-in-oil-in-water emulsion resulted in enhanced mucosal and systemic immune responses upon intranasal and oral administration of ovalbumin in mice (Shahiwala and Amiiji, 2008). However, species and emulsion effects are plausible.

Conclusion

Further research is warranted to explore potential viable delivery mechanisms for intranasal administration of BVDV and other respiratory vaccine agents. The absence of detectable mucosal or systemic immune responses following intranasal administration of PRM suggests that this particular formulation may not be a promising intranasal vaccine candidate. It could be beneficial to conduct a similar study that evaluates PRM without inclusion of the squalene-based adjuvant, to assess the immunogenicity of the viral components without the hypothesized adjuvant interference and such a study would further explore the hypothesized adjuvant-associated immune interference.

Table 3.1. Description of vaccine treatment regimens.[1]

CON-PYR (n = 10)	**INF-BSG (n = 10)**	**PRM-PYR (n = 10)**
d 0: 1 mL/naris sterile saline, administered intranasal	d 0: 1 mL/naris Inforce 3, administered intranasal	d 0: 1 mL/naris Prism 5, administered intranasal
d 29: 2 mL Pyramid 5, administered parenteral	d 29: 2 mL Bovi-Shield Gold 5, administered parenteral	d 29: 2 mLPyramid 5, administered parenteral

[1]CON = saline administration, PYR = parenteral vaccination with Pyramid 5 (Boehringer Ingelheim Vetmedica, Inc., St. Joesph, MO), INF = intranasal vaccination with Inforce 3 (Zoetis, Kalamazoo, MI), BSG = parenteral vaccination with Bovi-shield GOLD 5 (Zoetis), PRM = intranasal vaccination with Prism 5 (Boehringer Ingelheim Vetmedica, Inc.).

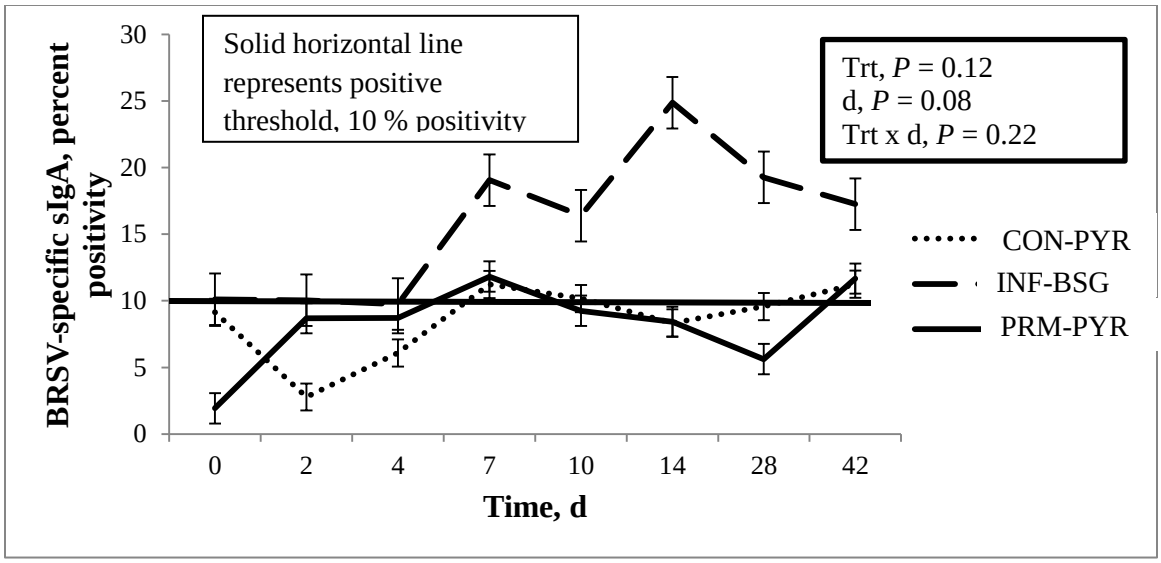

Figure 3.1. Effect of vaccine regimen on percent positivity index of BRSV-specific secretory IgA. CON-PYR = saline control on d 0, followed by parenteral administration of Pyramid 5 on d 29; INF-BSG = intranasal vaccination with Inforce 3 on d 0, followed by parenteral administration of Bovi-shield Gold 5 on d 29; PRM-PYR = intranasal vaccination with Prism 5 on d 0, followed by parenteral administration of Pyramid 5 on d 29.

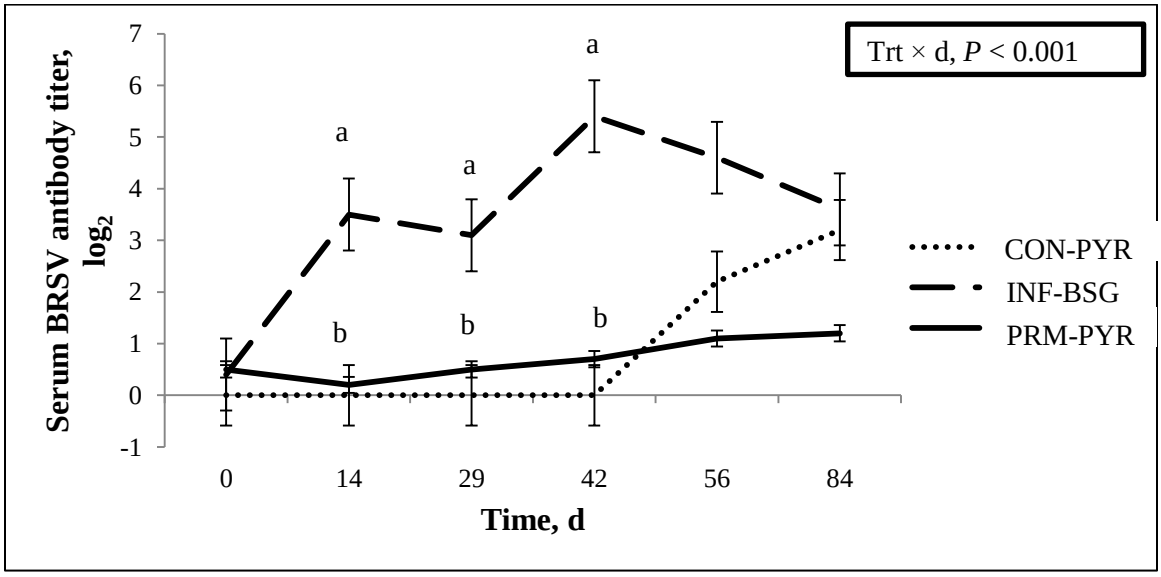

Figure 3.2. Effect of vaccine regimen on BRSV antibody titer concentration. Treatments with unlike letters differ, P≤0.06. CON-PYR = saline control on d 0, followed by parenteral administration of Pyramid 5 on d 29; INF-BSG = intranasal vaccination with Inforce 3 on d 0, followed by parenteral administration of Bovi-shield Gold 5 on d 29; PRM-PYR = intranasal vaccination with Prism 5 on d 0, followed by parenteral administration of Pyramid 5 on d 29.

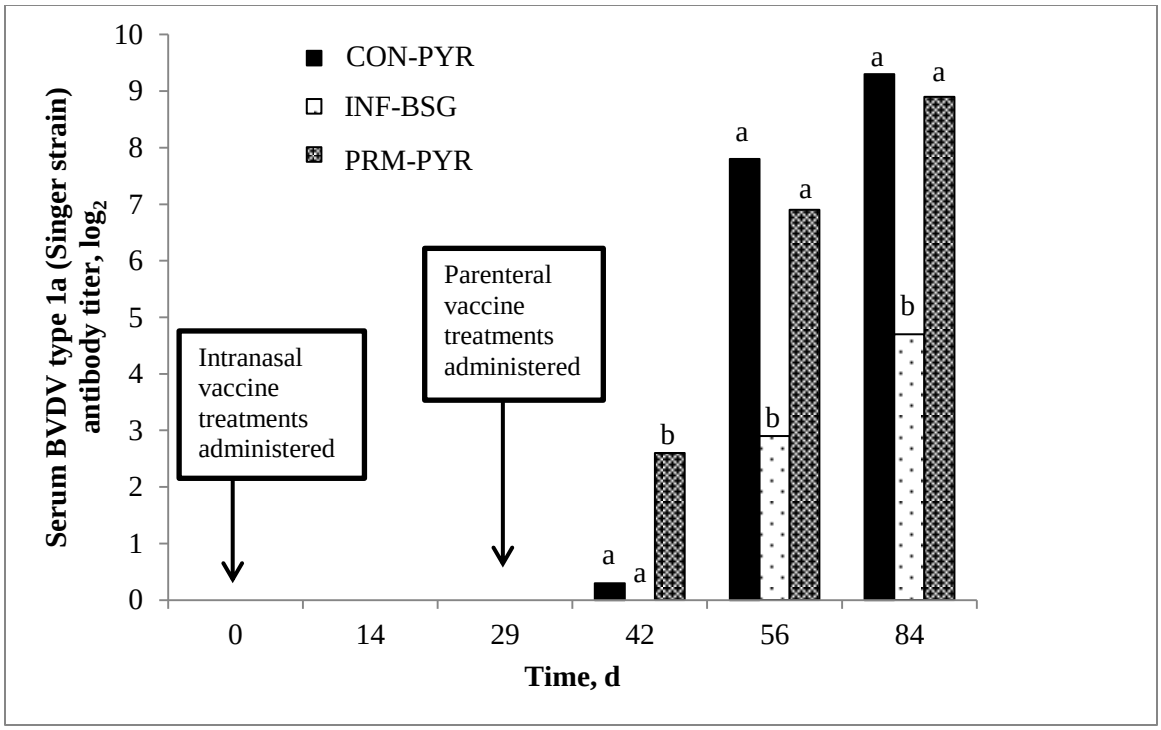

Figure 3.3. Effect of vaccine regimen on serum BVDV type 1a (Singer strain) antibody titer concentration. Treatments with unlike letters differ, P<0.05. CON-PYR = saline control on d 0, followed by parenteral administration of Pyramid 5 on d 29; INF-BSG = intranasal vaccination with Inforce 3 on d 0, followed by parenteral administration of Bovi-shield Gold 5 on d 29; PRM-PYR = intranasal vaccination with Prism 5 on d 0, followed by parenteral administration of Pyramid 5 on d 29.

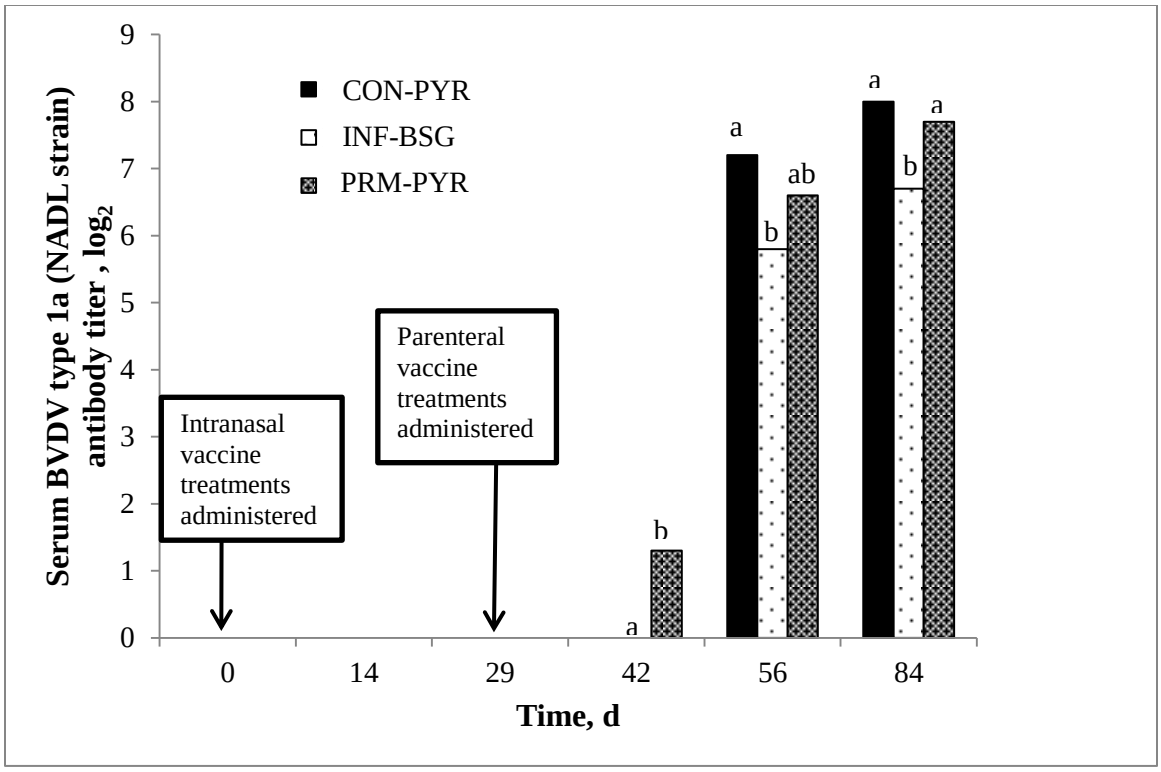

Figure 3.4. Effect of vaccine regimen on serum BVDV type 1a (NADL strain) antibody titer concentration. Treatments with unlike letters differ, $P<0.05$. CON-PYR = saline control on d 0, followed by parenteral administration of Pyramid 5 on d 29; INF-BSG = intranasal vaccination with Inforce 3 on d 0, followed by parenteral administration of Bovi-shield Gold 5 on d 29; PRM-PYR = intranasal vaccination with Prism 5 on d 0, followed by parenteral administration of Pyramid 5 on d 29.

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CHAPTER IV

SERUM LEUKOTOXIN ANTIBODY RESPONSE IN BEEF CALVES ADMINISTERED DIFFERENT COMBINATION VIRAL-BACTERIAL RESPIRATORY VACCINES

H. D. Hughes

West Texas A&M University

ABSTRACT

A total of 35 clinically healthy, crossbred beef steer calves weighing 178 ± 24 kg were stratified by pre-trial serum antibody against *Mannheimia haemolytica* (*Mh*) whole cell wall then assigned randomly to 1 of 3 vaccine treatment regimens consisting of: 1) sentinel control (CON); n=5 (no vaccine/toxoid administered), 2) Pyramid 5 + Presponse SQ (PRE); n=15 or 3) Bovi-shield Gold One Shot (ONE); n=15). Pen assignment was such that 3 calves from PRE and ONE treatments, and one sentinel CON were represented in each of 5 study pens, with animal as experimental unit (7 calves/pen). Serum antibody concentrations against *Mh* leukotoxin (Lkt) were evaluated from d 0, 7, 14, 28, 42 and 56, and a treatment × d interaction was observed ($P < 0.001$). On d 7, 14, and 28, cattle administered either of the vaccines had markedly increased Lkt antibody concentrations compared to CON. On d 42, Lkt antibody concentrations in cattle on the ONE treatment were higher ($P = 0.05$) than CON, but did not differ ($P = 1.00$) from cattle receiving the PRE vaccine. Administration of either combination vaccine in lightweight beef steers produced similar efficacy, as indicated by similar Lkt antibody response.

Introduction

Mannheimia haemolytica (*Mh*) is the major bacterial specie involved in the pathogenesis of bovine respiratory disease (BRD) in beef cattle (Frank, 1986). Further, the most important cause of BRD mortality is fibrinous bronchopneumonia attributable to *Mh* (Cavirani et al., 2007). In healthy cattle, *Mh* bacteria are present in the tonsillar crypt and exist as commensal microbial flora; however, during stress- and/or viral-induced immune dysfunction, these bacteria can rapidly proliferate, colonize the nasopharynx, and enter the lungs via aerosolized droplets (Thumbikat et al., 2005).

Leukotoxin (Lkt) is secreted from the bacteria during this growth phase, and stimulates serum antibody responses during both natural exposure and Lkt-containing vaccine exposure (Srinand et al., 1996). Typically, the proliferation of *Mh*, and subsequent release of Lkt, follows viral challenge, making the use of vaccination against the common respiratory viruses important and lends justification for the use of such agents in combination with bacterin/toxoid product formulations. However, multi-antigen immune interference has been hypothesized and warrants further investigation.

Different respiratory viral vaccine products possess unique characteristics, such as various antigen concentration, specific strains of killed and/or live-attenuated virus, and presence or absence of an adjuvant. Similarly, bacterial and toxoid components within vaccines can vary. Early bacterin products were developed from live cultures of *Bacillus boviseptica*, which has since been taxonomically divided into *Mh* and *P. multocida*. Many of these early bacterins were ineffective (Miller et al., 1927) or detrimental (Farley, 1932) in the clinical setting. Likewise, in more recent studies, *Mh* bacterins have demonstrated no benefit (Hamdy et al., 1965; Martin, 1983) or were detrimental

(Schipper and Kelling, 1971; Friend et al., 1977; Wilkie et al., 1980). These bacterins are derived from whole cell cultures, which are live-attenuated; therefore, these may be more immunogenic, hence the detrimental effects in multiple studies. The bacterial component in the Bovi-shield GOLD One Shot (ONE; Zoetis, Kalamazoo, MI) vaccine-bacterin evaluated in this study is derived from whole cell *Mh* cultures and is delivered alongside a sterile diluent (Zoetis, 2015).

The importance of Lkt in the pathogenesis of *Mh* infection is well established (Shewen and Wilkie, 1982, 1985; Gentry et al., 1985) and commercial bacterins have been developed containing cell-free *Mh* A1 culture supernatant, which contains the Lkt produced during logarithmic-phase growth of *Mh* cell culture (Shewen and Wilkie, 1988; Shewen et al., 1988). These bacterins are considered "subunit-enriched" vaccines because they capitalize on various subunits associated with *Mh* logarithmic-phase growth. Experimental trials using these Lkt-containing bacterins demonstrated improved efficacy over previous bacterin products (Shewen et al., 1988; Bateman, 1988; Jim et al., 1988; Thorlakson et al., 1990).

Since then, various pharmaceutical companies have developed similar subunit bacterins which consist of various outer membrane protein extracts (Srinand et al., 1996), including toxoids, which contain toxins secreted by bacteria that have been inactivated via formalin treatment. The inactivated toxin is safe in the host, yet stimulates active immunity against virulent Lkt for anticipated future exposure (WHO, 2015). The *Mh* component in the Pyramid 5 + Presponse SQ (PRE; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) vaccine-bacterin used in this study is a Lkt toxoid, delivered alongside a proprietary adjuvant (Boehringer Ingelheim Vetmedica, Inc, 2015).

Presumably, the adjuvant serves to enhance the immunogenicity of the vaccine-bacterin, since the toxoid component is inactivated, much like a killed viral vaccine. The two combination vaccines in this study are each designed to stimulate immunity against *Mh* Lkt using different molecular components; therefore, the objective of this study was to compare the serum Lkt antibody response in cattle vaccinated with 2 different pentavalent modified live virus (MLV) vaccine-bacterin products, (PRE vs. ONE) each containing unique Lkt antibody-stimulating agents.

Materials and Methods

General

Animal methods and procedures were approved by the IACUC committee at West Texas A&M University (WTAMU). The PRE and ONE vaccine-bacterins used in the study were acquired from Hereford Veterinary Supply (Hereford, TX). Appropriate vaccine handling and administrative procedures were followed using aseptic technique.

A total of 35 (n = 10, each vaccine treatment; n = 5, sentinel control treatment) clinically healthy, crossbred beef steer calves were stratified by pre-trial serum antibody against *Mh* whole cell wall as described by Confer et al. (1998), then assigned randomly to 1 of 3 vaccine treatment regimens (Table 3.1) consisting of 1) sentinel control (CON); n=5 (no vaccine/toxoid administered), 2) PRE; n=15 (Boehringer Ingelheim Vetmedica, St. Joseph, MO) or 3) ONE; n=15 (Zoetis, Kalamazoo, MI). Both vaccine-bacterin products evaluated in this study contained 5 respiratory virus agents (bovine herpesvirus-1, bovine viral diarrhea virus type 1 and 2, bovine respiratory syncytial virus, and parainfluenza-3 virus), yet the specific strains included differ slightly. Likewise, the

bacterin component in each product differs. The Lkt antigenic component of PRE is inactivated toxoid alongside a proprietary adjuvant; whereas, the Lkt antigenic component of ONE is comprised of whole cell *Mh* cultures propogated to increase production of Lkt and capsular and cell-associated antigens, alongside a sterile diluent.

Blood was collected 28 d prior to the beginning of the trial to facilitate subset determination; 35 steers from a pool of 41 with the lowest pre-trial *Mh* cell wall antibody concentration were used according to data from an ELISA conducted at the laboratory of Dr. Anthony Confer at Oklahoma State University College of Veterinary Medicine (Stillwater, OK). Calves had low or no antibody against *Mh* whole cell wall; nevertheless, they were stratified and assigned to treatment according to pre-trial *Mh* cell wall antibody concentration to ensure equivalent concentration between the experimental treatments.

Vaccine-bacterin treatments were administered to the appropriate treatment groups on d 0 and assigned randomly to isolated pens at the WTAMU Research Feedlot. Pen assignment was such that 3 calves from Treatments 1 and 2, and one sentinel control were represented in each of 5 study pens (7 calves/pen). Calves were allowed *ad libitum* access to water and a common starter ration during the 56-d study. Serum antibody concentrations against *Mh* Lkt were evaluated from d 0, 7, 14, 28, 42, and 56.

Sample collection and assay procedures

Blood samples were collected via jugular venipuncture from all animals at approximately 0700 beginning on d 0 (06 Nov 2013), and on the appropriate sampling d through d 56 (01 Jan 2014). To determine serum concentrations of *Mh* LKT antibody titers, blood was collected into blood collection tubes with no additive (Vacutainer®

SST, 10 mL tube, Ref # 367985, Becton Dickinson, Franklin Lakes, NJ). Whole blood was allowed to clot for at least 30 min and serum was collected following centrifugation at 1,250 × g for 20 min at 20° C.

Serum was stored in triplicate aliquots at -20° C until subsequent analysis, where serum from each animal collected on study d 0, 7, 14, 28, 42, and 56 was packaged on ice and shipped via next d parcel service to the laboratory of Dr. Anthony Confer at Oklahoma State University College of Veterinary Medicine (Stillwater, OK) for quantification of serum antibody concentration against *Mh* Lkt**.**

Leukotoxin serology

Antibodies to *Mh* whole cells and to Lkt were determined by enzyme-linked immunosorbent assay (ELISA; Confer et al., 1997; Confer et al., 1998). The *Mh* A1 strain used for antigen preparation was originally isolated from a feedlot calf (Panciera and Corstvet, 1984). Formalinized *Mh* was prepared from a washed 24-hr culture by suspending cells in 0.4% formalinized saline at a concentration determined spectrophotometrically to be 1.850 OD650.

The Lkt was prepared from culture supernatant from a 3-hr culture of *Mh* A1 grown in RPMI-1640 medium at 37 °C in a shaking incubator. The Lkt was partially purified by precipitation with 40-60% ammonium sulfate as previously described (Clinkenbeard et al., 1994). The precipitate was re-suspended in 3M guanidine containing 59 mM NaHPO4 and 100 mM NaCl. By SDS-PAGE of the Lkt preparation, one intensely staining band was identified at 105 kDa and confirmed to be Lkt on a western blot using an anti-Lkt monoclonal antibody (Confer et al., 1998).

Leukotoxic activity was 104 Lkt Units per ml (Clinkenbeard et al., 1994). The 2-keto-3-deoxyoctonate concentration was 7.5 μg per mg of protein (Osborn, 1963). Wells of 96-well microtiter plates were coated with whole cells at an optical density reading equivalent to 108 CFU of a 24-hr culture or with Lkt at 50 ng per well. Sera were diluted in PBS-Tween 20 containing 1% BSA and tested at dilutions of 1:800 for whole cells and 1:1600 for Lkt. The extent of antibody binding was detected using a 1:400 dilution of horseradish peroxidase-conjugated, affinity purified rabbit anti-bovine IgG. Antibody responses as indicated by colorimetric intensity were read at OD490. Results of OD490 were extrapolated to reflect ng of IgG/well based on a standard curve of known IgG concentrations for each plate.

Statistical analyses

The *Mh* Lkt antibody concentration data were analyzed using PROC MIXED procedure of SAS (SAS Inst., Cary, NC) with repeated measures in a completely randomized design with animal as the experimental unit. The repeated statement was d, and effects of treatment, d, and their interaction were evaluated. The covariance structure used was unstructured and the Kenward-Roger degree of freedom method was employed. Differences of least squares means were adjusted and analyzed using the Tukey-Kramer method in SAS with statistical significance established for a P-value ≤ 0.05 and a tendency was considered for a P-value between 0.06 and 0.10.

Results and Discussion

Graphical representation of the Lkt antibody concentration is illustrated in Figure 3.1. On d 0, all treatments had very low Lkt antibody, and did not differ ($P = 1.00$). A treatment × d interaction was observed ($P < 0.001$); on d 7, 14, and 28 cattle administered

either of the vaccines had greater ($P \leq 0.01$) Lkt-specific antibody concentration than CON. The Lkt antibody concentration for ONE tended ($P = 0.085$) to be greater than PRE on d 14. On d 42, the Lkt-specific antibody concentration for ONE was greater ($P = 0.03$) than CON cattle, but did not differ ($P = 0.87$) from cattle receiving the PRE vaccine-bacterin. For either vaccine-bacterin group, anti-Lkt antibody concentration increased transiently (D effect; $P < 0.001$); the greatest concentrations were observed on d 14, post-vaccination, and decreased thereafter. This may suggest a relatively short duration of Lkt-specific humoral immunity for either vaccine-bacterin. On d 56, no differences in Lkt antibody concentration ($P \geq 0.27$) were observed between vaccinates or CON. Overall, the Lkt antibody concentrations were numerically greater for ONE, but did not differ statistically ($P \geq 0.09$) from calves administered PRE.

Various studies have demonstrated an immune response to parenteral administration of Lkt-containing bacterin products (Shewen and Wilkie, 1988; Confer et al., 1997; Fulton et al., 2004). Furthermore, experimental challenge models and commercial field studies have demonstrated that using *Mh* bacterins in cattle results in lower BRD morbidity rates (Jim et al., 1988; Rice et al., 1995). Within these previous studies, the bacterial and toxoid components in the vaccines vary. Confer et al. (1998) vaccinated weanling beef cattle with ONE or PRE and observed equivalent anti-Lkt responses between the two vaccines, whereas, ONE elicited a greater antibody response to *Mh* whole cell wall. It is important to note that the PRE used in this trial was an earlier prototype than the PRE used in the current study, and the differences between these two PRE prototypes is proprietary. In this study, the Lkt antigenic component of PRE is leukotoxin-toxoid, inactivated by formalin, which is delivered alongside a

proprietary adjuvant; whereas, the Lkt antigenic component of ONE is comprised of whole cell cultures of *Mh* (Zoetis, 2015). It is unclear if the numerical difference in Lkt antibody response between the two vaccine-bacterins is biologically significant (i.e., results in a clinical difference in protection from natural pathogen challenge); nevertheless, the different bacterial components of the two vaccine-bacterins compared could explain the numerical difference observed in our study. Whole cell cultures in the ONE vaccine may be more immunogenic than the toxoid component in the PRE vaccine. It is likely that the PRE vaccine contains the proprietary adjuvant to overcome the less immunostimulatory nature of inactivated toxoid.

Conclusion

The PRE and ONE vaccine-bacterins compared in the current study are intended to protect against the same viruses in addition to containing unique Lkt antibody stimulating components which provide immunity against *Mh*. In this trial using healthy beef calves initially naïve to *Mh*, either vaccine treatment resulted in marked increases in Lkt antibody compared to CON. The ONE treatment had numerically greater Lkt-specific antibody post-vaccination compared to PRE, which may be explained by the differences in bacterin composition. Additional research involving both controlled *Mh* challenge models and large-pen studies conducted in the commercial feedlot setting to evaluate differences in clinical BRD morbidity are necessary to elucidate whether the numerical differences in Lkt-specific antibody response are biologically significant.

Table 4.1. Description of vaccine treatment regimens[1]

CON (n = 5)	PRE (n = 15)	ONE (n = 15)
Sentinel Control	Pyramid 5 + Presponse SQ	Bovi-shield Gold One Shot
No vaccine administered	2 ml administered subcutaneously in the neck on d 0	2 ml administered subcutaneously in the neck on d 0

[1]Treatments consisted of sentinel control (CON), administration of Pyramid 5 + Presponse SQ (PRE; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO), and Bovi-shield GOLD One Shot (ONE; Zoetis, Kalamazoo, MI).

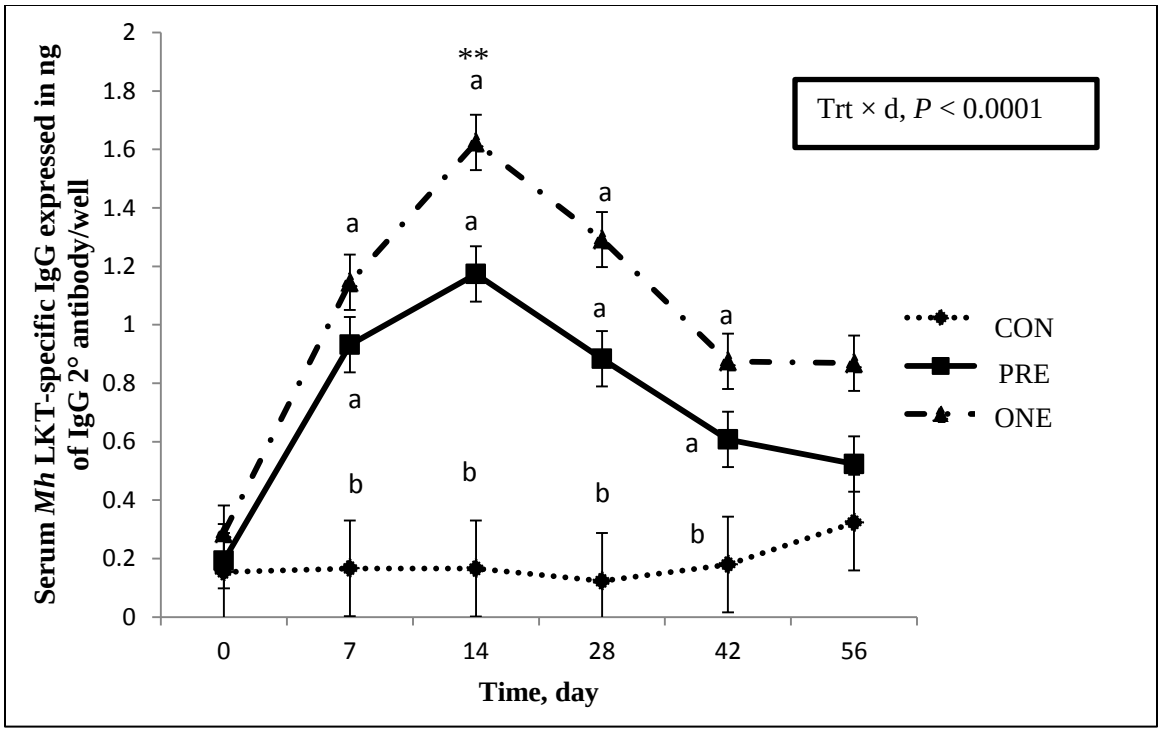

Figure 4.1. Effect of vaccine regimen on *M. haemolytica* LKT serum antibody concentration. Treatments with unlike letters differ ($P < 0.05$) via LSD mean separation. **PRE and ONE tend to differ ($P = 0.09$) via Tukey-Kramer mean separation. Treatments consisted of: PRE = Pyramid 5 + Presponse SQ administered on d 0; ONE=Bovi-Shield Gold One Shot administered on d 0; CON = sentinel control.

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CHAPTER V

EFFECT OF DIFFERENT COMBINATION VIRAL-BACTERIAL RESPIRATORY VACCINES ON SERUM LEUKOTOXIN ANTIBODY, ACUTE PHASE RESPONSE, AND PERFORMANCE IN BEEF HEIFER CALVES

H. D. Hughes

West Texas A&M University

ABSTRACT

Combination vaccines which contain viral and bacterial agents are designed to prevent bovine respiratory disease (BRD) and multivalent vaccines are increasingly used in beef cattle. A total of 30 clinically healthy beef heifer calves were stratified by pre-trial serum antibody against *Manheimia haemolytica* (*Mh*) whole cell wall, then assigned randomly to 1 of 3 vaccine treatment regimens consisting of: 1) Pyramid 5 + Presponse SQ (PRE); n=10 (Boehringer Ingelheim Vetmedica, St. Joseph, MO), 2) Bovi-shield Gold One Shot (ONE); n=10 (Zoetis, Kalamazoo, MI), or 3) sentinel control (CON); n=10 (no vaccine/toxoid administered). The heifers resided in a single pen, with individual animal serving as experimental unit. Serum antibody concentrations against *Mh* leukotoxin (Lkt) were evaluated from d 0, 4, 7, 14, 28, 42 and 56, and a treatment × d interaction was observed ($P < 0.001$). Cattle administered either of the vaccines had greater Lkt antibody concentrations than CON on d 14 and 28 ($P \leq 0.04$), and cattle administered ONE were greater than CON on d 7 ($P = 0.03$) but did not differ from PRE ($P = 0.49$). No differences were observed in rectal temperature or average daily gain. Respiratory vaccination resulted in a detectable acute phase response, as evidenced by an increase in serum haptoglobin (Hp) concentration; PRE exhibited greater Hp concentrations than CON ($P = 0.01$) but was not different from ONE ($P = 0.53$). Haptoglobin was numerically greater on d 7 in cattle receiving PRE compared to the ONE and CON animals. However, no effect of vaccine regimen on serum ceruloplasmin concentration was observed ($P = 0.88$).

Introduction

Combination viral-bacterial vaccines may serve as an effective preventative measure against bovine respiratory disease (BRD) when administered to cattle, particularly since these products contain *Manheimia haemolytica* (*Mh*) agents; the primary bacteria implicated in BRD (Frank, 1986).

Vaccination of newly received cattle is a common practice in the feedlot, yet this practice may contribute to an acute inflammatory response by stimulating hepatic production of acute phase proteins (APP), such as haptoglobin (Hp) or ceruloplasmin (Cp), while also inducing fever (Baumann and Gauldie, 1994). The resulting alteration in metabolism, during what is called an acute phase response (APR), could also transiently reduce animal performance (Johnson, 1997; Klasing and Korver, 1997). The APR is a major component of the phenomenon known in the cattle industry as "vaccine sweats".

In cattle, it is understood that Hp binds to free-hemoglobin in the blood to form a Hp:hemoglobin complex, thereby restricting the availability of iron required for bacterial growth (Eaton et al., 1982). Haptoglobin is the most widely assayed APP in cattle because it is virtually undetectable in unstressed, homeostatic cattle, yet increases rapidly in response to stress and inflammation (Connor and Eckersall, 1988).

Ceruloplasmin is another APP synthesized during inflammation and serves to transport copper and ferrous iron to tissues throughout the body during an acute phase response (McCord and Fridovich, 1968). However, unlike Hp, it can be present in stressed and unstressed cattle alike due to nutritional status, making increases during inflammation less profound (Arthington et al., 2003; Arthington et al., 1996).

Data which elucidate the effect of vaccination on the acute phase response in cattle are limited. Stokka et al. (1994) administered various clostridial vaccines and observed significantly greater Hp concentrations in both vaccinated groups compared to unvaccinated controls. Similar to the current study, Arthington et al. (2013) administered One Shot to 12 heifers, alongside 11 unvaccinated controls, and observed greater plasma Hp and Cp on d 1, 3, 6, 9, and 12 following vaccination. Additionally, average daily gain (ADG) was greater ($P \leq 0.05$) for controls versus vaccinated heifers.

The objective of the current study was to observe the effects of different commercially available vaccine-bacterins on the Lkt-specific antibody and APR (i.e., indicated by Hp, Cp, and rectal temperature).

Materials and Methods

General

Animal methods and procedures were approved by the IACUC committee at West Texas A&M University (WTAMU). The vaccine products used in the study were acquired from Hereford Veterinary Supply (Hereford, TX). Appropriate vaccine handling and administrative procedures were followed using aseptic technique.

A total of 30 (n = 10) clinically healthy, crossbred beef heifer calves were stratified by pre-trial serum antibody against *Mh* whole cell wall as described by Confer et al. (1998), then assigned randomly to 1 of 3 vaccine treatment regimens (Table 5.1) consisting of: 1) Pyramid 5 + Presponse SQ (PRE); n=10 (Boehringer Ingelheim Vetmedica, St. Joseph, MO), 2) Bovi-shield Gold 5 One Shot (ONE); n=10 (Zoetis, Kalamazoo, MI), or 3) sentinel control (CON); n=10 (no vaccine-bacterin administered).

Blood was collected prior to the beginning of the trial and analyzed via the ELISA procedure at the laboratory of Dr. Anthony Confer at Oklahoma State University College of Veterinary Medicine (Stillwater, OK). Calves were stratified and assigned randomly to treatment according to pre-trial *Mh* cell wall antibody concentration to ensure equivalent concentration between experimental treatments.

Vaccine-bacterins were administered to the appropriate treatment groups on d 0 and all heifers on study were placed together in a single pen at the WTAMU Research Feedlot. Calves were allowed *ad libitum* access to water and a common ration during the 56-d study. A daily clinical illness score was recorded each morning (0700), but there was neither clinical illness nor mortalities observed during this study.

Sample collection and assay procedures

Blood samples were collected via jugular venipuncture from experimental animals at approximately 0500 beginning on d 0 (24 Jun 2014), 4, 7, 14, 28, 42, and 56 (study ended on 19 Aug 2014). To determine serum concentrations of *Mh* LKT antibody titers, blood was collected into evacuated blood collection tubes with no additive (Vacutainer®, 10 mL tube, Ref # 367985, Becton Dickinson, Franklin Lakes, NJ). Whole blood was allowed to clot for at least 30 min and serum was collected following centrifugation at 1,250 × g for 20 min at 20° C.

Serum was stored in aliquots at -20° C until subsequent analysis, where serum from each animal collected from designated study d (0, 4, 7, 14, 28, 42, 56) was packaged on ice and shipped via next d parcel service to the laboratory of Dr. Anthony Confer at Oklahoma State University for quantification of serum antibody concentration again *Mh* LKT**.**

M. haemolytica serology

Antibodies to *Mh* whole cells and to Lkt were determined by enzyme-linked immunosorbent assays (ELISAs) (Confer et al., 1997; Confer et al., 1998). The *Mh* A1 strain used for antigen preparation was originally isolated from a feedlot calf (Panciera and Corstvet, 1984). Formalinized *Mh* was prepared from a washed 24-hr culture by suspending cells in 0.4% formalinized saline at a concentration determined spectrophotometrically to be 1.850 OD650.

The Lkt was prepared from culture supernatant from a 3-hour culture of *Mh* A1 grown in RPMI-1640 medium at 37 ^{o}C in a shaking incubator. The Lkt was partially purified by precipitation with 40-60% ammonium sulfate as previously described (Clinkenbeard et al., 1994). The precipitate was re-suspended in 3M guanidine containing 59 mM NaHPO4 and 100 mM NaCl. By SDS- PAGE of the Lkt preparation, one intensely staining band was identified at 105 kDa and confirmed to be Lkt on a western blot using an anti-Lkt monoclonal antibody (Confer et al., 1998).

Leukotoxic activity was 104 Lkt Units per ml (Clinkenbeard et al., 1994). The 2-keto-3-deoxyoctonate concentration was 7.5 μg per mg of protein (Osborn, 1963). Wells of 96-well microtiter plates were coated with whole cells at an optical density reading equivalent to 108 CFU of a 24-hr culture or with Lkt at 50 ng per well. Sera were diluted in PBS-Tween 20 containing 1% BSA and tested at dilutions of 1:800 for whole cells and 1:1600 for Lkt. The extent of antibody binding was detected using a 1:400 dilution of horseradish peroxidase-conjugated, affinity purified rabbit anti-bovine IgG. Antibody responses as indicated by colorimetric intensity were read at OD490. Results of OD490

were extrapolated to reflect ng of IgG/well based on a standard curve of known IgG concentrations for each plate.

Haptoglobin and ceruloplasmin serology

Concentrations of serum Hp were determined using a bovine-specific ELISA assay (Immunology Consultants Laboratory, Inc., Newburg, OR). The Hp present in samples reacts with the anti-Hp antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti-Hp antibodies conjugated with horseradish peroxidase (HRP) are added. These enzyme-labeled antibodies form complexes with the previously bound Hp. Following another washing step, the enzyme bound to the immunosorbent was assayed by the addition of a chromogenic substrate. The quantity of bound enzyme is directly proportional to the concentration of Hp in the test sample, and was quantified by reading the absorbance at 450 nm. The quantity of Hp in the test sample was then interpolated from the standard curve constructed from the standards, then corrected for sample dilution.

Concentrations of serum Cp were determined using a colorimetric activity assay kit (Arbor Assays, Ann Arbor, MI). The Cp was quantitatively measured using a Cp standard provided in the kit which generates a standard curve. Samples were appropriately diluted in the provided assay buffer and added to the wells of a half area clear plate. The reconstituted Cp Substrate is added and the plate is incubated at 30 °C for 60 mins. The Cp in the samples reacts with the substrate to produce a colored product. The OD is read at 560 nm, and results were expressed in terms of milliunits of CP activity per mL.

Additionally, rectal temperature was determined on d 0, 4, 7, and 14 using a hand held digital thermometer.

Statistical analyses

Dependent variables were analyzed using PROC MIXED (SAS Inst., Cary, NC) with repeated measures in a completely randomized design with animal serving as the experimental unit. The repeated statement was d, and effects of treatment, d, and their interaction were evaluated. The Kenward-Roger degree of freedom method was employed to account for unbalanced samples. Differences of least squares means were first analyzed using the pdiff option in SAS. The Lkt antibody data was also adjusted and analyzed using the Tukey-Kramer test in SAS. Statistical significance was established when a *P*-value was ≤ 0.05 and tendencies were considered between 0.06 and 0.10.

Results and Discussion

Leukotoxin antibody response

Antibodies against *Mh* Lkt are illustrated in Figure 1. On d 7, cattle receiving ONE had greater antibody concentration than CON ($P=0.03$), but did not differ from PRE ($P=0.49$). Cattle administered either of the vaccines had greater Lkt antibody concentrations on d 14 and 28 compared to control ($P<0.04$); however, following the Tukey-Kramer adjustment, antibody concentration only tended to differ between vaccine treatments and control treatment on d 14 and 28 ($P\leq0.098$). These results are consistent with a previous study conducted at WTAMU, where both vaccines had greater Lkt antibody concentrations versus control cattle on d 7, 14, and 28 (Chapter II). It is important to note that the baseline Lkt antibody concentrations on d 0, in this study, were

markedly greater compared to the previous study composing Chapter II (~ 1.0 ng IgG vs. ~ 0.2 IgG).

Acute phase response

Serum Cp increased transiently ($P < 0.001$), peaking at d 7 and returning to baseline at d 14; however, there was no effect of vaccine treatment on Cp concentration. Haptoglobin was different between treatments ($P = 0.01$), but there was no treatment × d interaction ($P = 0.26$) evident. Results are represented in Figures 5.2 and 5.3. Serum Hp concentration was increased for PRE vs. CON ($P = 0.01$), but was not different from ONE ($P = 0.53$). Serum concentrations of Hp were numerically greater in PRE vs. ONE and CON cattle on d 7 (304,247, 138,303, and 108,044 ng/mL, respectively) but no treatment × d interaction was evident ($P = 0.26$). These results may suggest a greater inflammatory response induced by PRE; however, it is not clear whether this observation has clinically meaningful impact in terms of host response.

Treatment did not affect ($P = 0.85$) rectal temperature during the first 14 d following vaccination (Table 5.2). Ambient temperature on d 0 was particularly warm and humid. Likely due to the ambient conditions, body temperatures were relatively high across treatments on d 0, with rectal temperature averaging 39.2° C. The increased temperatures observed for all heifers on d 0 could have made effects of treatment on the febrile response following vaccination difficult to detect.

Performance

Average daily gain was assessed for various time points across the study, including between d 0 and 7, d 0 to 14, d 7 to 14, and d 0 to 56. No differences in performance were observed for any of these interim periods (Table 5.3;$P \geq 0.33$).

In contrast to results in Chapter IV, PRE and ONE produced a similar Lkt antibody response and the magnitude of difference compared to CON was less. The initial concentration of Lkt antibody was much greater during vaccination on d 0 for the current study. This likely blunted the Lkt antibody response to the combination vaccines evaluated in the current study.

The acute phase response (APR) following respiratory vaccination, as indicated by serum Hp concentration, was evident for vaccinates compared to CON. However, the magnitude of the APR did not clearly differ between the 2 vaccine-bacterins compared (PRE vs. ONE) based on the variables currently measured (i.e., rectal temperature, Cp, and Hp). The overall Hp concentration in PRE cattle was greater compared to CON (P = 0.02), but ONE was intermediate and did not differ from PRE or CON ($P \geq 0.22$). The numerical increase in serum Hp observed for PRE might suggest that PRE induces a greater APR than ONE; however, additional research is needed to clearly distinguish this.

If PRE does indeed induce greater inflammation, it is not clear whether it crosses the threshold of being clinically detrimental. The heifers in this study were observed to be healthy throughout, and results would suggest that the numerical differences observed in Hp between vaccines had no relevant biological or production impact. Finally, performance data did not indicate an effect of vaccine treatment that would substantiate any evidence that vaccine treatment altered production.

Conclusion

Few data exist which elucidate the impacts of administering a combination viral-bacterial vaccine on the APR in beef cattle. Further research is necessary to determine what differences in inflammation may exist following vaccination with either PRE or

ONE, and whether or not any potential variation between the two vaccines has a detrimental impact on health or performance.

Table 5.1. Description of vaccine treatment regimens.[1]

PRE	ONE	CON
Pyramid 5 + Presponse SQ	Bovi-shield Gold One Shot	Sentinel Control
2 mL administered s.q. in the neck on d 0	2 mL administered s.q. in the neck on d 0	No vaccine administered

[1]Treatments consisted of administration of Pyramid 5 + Presponse SQ (PRE; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO), Bovi-shield GOLD One Shot (ONE; Zoetis, Kalamazoo, MI), and sentinel control (CON).

Table 5.2. Effect of vaccine treatment regiment on rectal temperature

	PRE[a]	ONE	CON	SEM	*P*-value
0[b]	39.58[c]	39.97	39.82	0.15	0.85
1	39.65	39.69	39.51	-	-
2	39.56	39.61	39.57	-	-
4	39.43	39.26	39.57	-	-
7	39.04	39.12	39.08	-	-
14	39.25	39.25	39.11	-	-

[a] Pyramid 5 + Presponse SQ administered on d 0; ONE = Bovi-Shield Gold 5 One Shot administered on d 0; CON = sentinel control.
[b] D
[c] Average rectal temperature across treatment, ℃

Table 5.3. Effect of vaccine treatment regimen on ADG, kg/d

	PRE[a]	ONE	CON	SEM	*P*-value
ADG, kg/d[b]					
d 0 to 7	0.35	0.27	0.68	0.55	0.46
d 0 to 14	0.86	0.82	0.99	0.21	0.33
d 7 to 14	1.33	1.27	1.22	0.55	0.95
d 0 to 56	1.17	1.13	1.23	0.12	0.34

[a] PRE = Pyramid 5 + Presponse SQ administered on d 0; ONE = Bovi-Shield Gold 5 One Shot administered on d 0; CON = sentinel control.
[b] Average daily gain across treatment

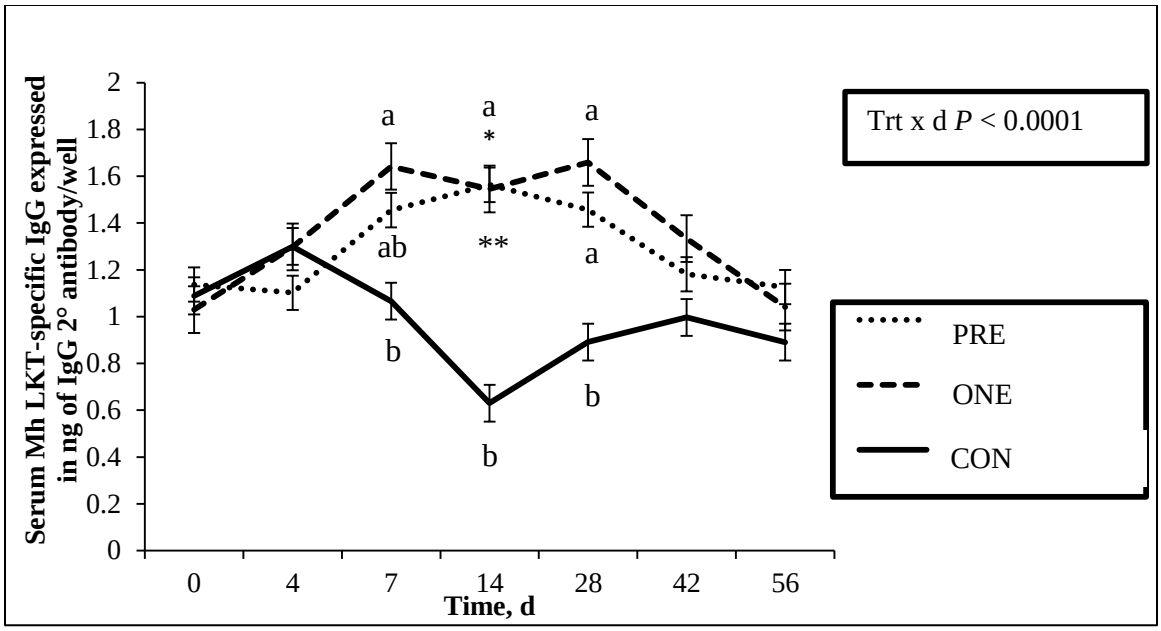

Figure 5.1. Effect of vaccine regimen on M. haemolytica LKT serum antibody concentration. *PRE and CON tend to differ, *P*=0.082 (Tukey Kramer adjustment). ** ONE and CON tend to differ, *P* = 0.098. Treatments with unlike letters differ (LSD). Treatments consisted of: PRE = Pyramid 5 + Presponse SQ administered on d 0; ONE = Bovi-Shield Gold 5 One Shot administered on d 0; CON = sentinel control.

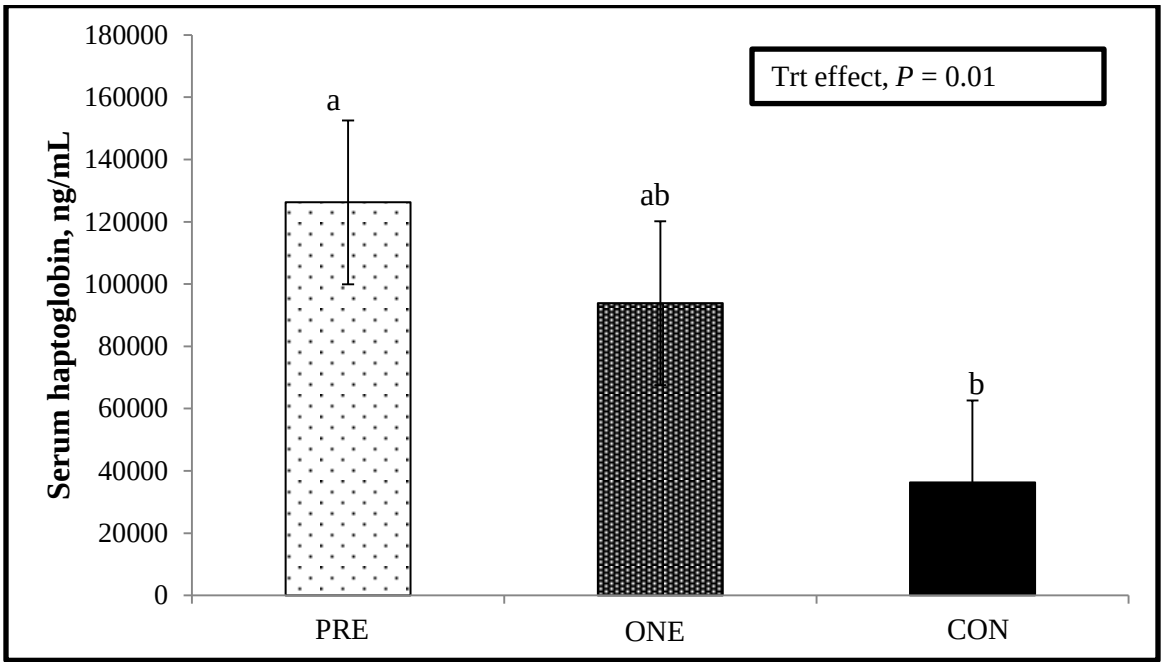

Figure 5.2. Effect of vaccine regimen on mean serum haptoglobin concentration. Treatments with unlike letters differ, *P* < 0.05. Treatments consisted of: PRE = Pyramid 5 + Presponse SQ administered on d 0; ONE = Bovi-Shield Gold 5 One Shot administered on d 0; CON = sentinel control.

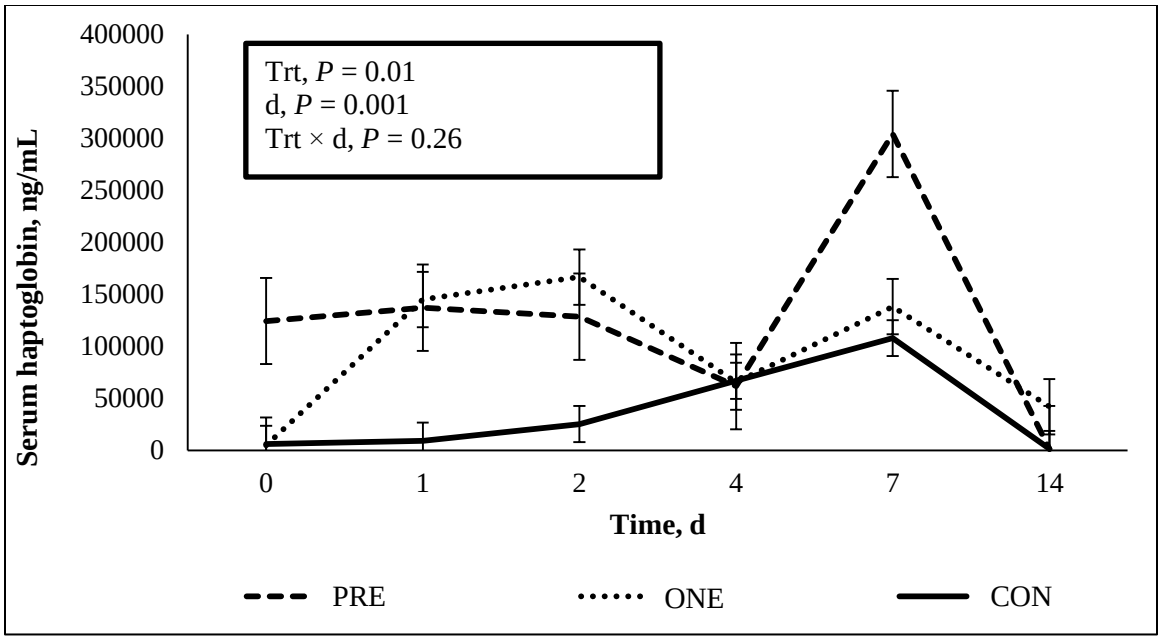

Figure 5.3. Effect of vaccine regimen on serum haptoglobin concentration. PRE=Pyramid 5 + Presponse; ONE=Bovi-Shield Gold 5 One Shot; CON=control.

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APPENDIX

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ABSTRACT

Activation of the innate immune system and acute phase response (APR) results in several responses that include fever, metabolic adaptations and changes in behavior. The APR can be modulated by many factors, with stress being the most common. An elevation of stress hormones for a short duration of time can be beneficial. However, elevation of stress hormones repeatedly or for an extended duration of time can be detrimental to the overall health and well-being of animals. The stress and APR responses can also be modulated by naturally-occurring variations, such as breed, gender, and temperament. These three natural variations modulate both of these responses, and can therefore modulate the ability of an animal to recover from a stressor or infection. Understanding that cattle have different immunological responses, based on naturally occurring variations such as these, may be the foundation of new studies on how to effectively manage cattle so that health is optimized and production is benefited.

Introduction

The acute phase response (APR) is defined as the body's early defense mechanism in response to trauma, inflammation or infection, and is facilitated by a cascade of systemic physiological reactions.[1–3] Initially, pro-inflammatory cytokines (i.e. TNF-a, IL-6 and IFN-g) are produced by activated leukocytes to induce the APR by stimulating changes in target cells to combat infection, while the location, type and combination of these proteins determine the final inflammatory outcome.[4–6] Typically, the response includes fever, changes in metabolism, and altered behavior accompa-nied by the hepatic production of various acute phase proteins (haptoglobin, serum amyloid A, C-reactive protein, fibrinogen and others) in response to trauma or infection. This non-specific component of immunity is defined as innate immunity, and serves as the first line of defense against bacterial, viral, protozoal or fungal pathogens.[7] Whether the APR is beneficial and appropriate to survival depends on the magnitude of the immunological challenge and the extent to which an animal's body can regulate the response to return to a homeostatic state.[8] Additionally, stress, which is defined as the state or perception of threatened homeo-stasis,[9] has been demonstrated to affect the APR through release of glucocorticoids that stimulate or inhibit, depending on the length of exposure, nearly all components of the innate immune response.[10,11] Stress hormones (i.e. the catecholamines and glucocorticoids) can generate an APR similar to that which occurs when an animal reacts to an invading pathogen or tissue injury and trauma;[12] therefore, stress hormones, such as cortisol, are important components to consider when observing characteristics of the APR. While the APR is essential for animal survival, it appears there are naturally occurring variations not only between animal species, but within species that serve as

modulators of innate immunity and the stress response. Differences in the APR attributed to gender, breed and temperament exist, and understanding these natural variations, in addition to potentially others, may allow for alternative management strategies to overcome barriers that these distinctions present.

Stress and the APR

The general concept that chronic stress has negative impacts on the health, well-being and productivity of animals is widely accepted among livestock producers and the scientific community alike. Stress in an animal can be indicated by transient increases in the hormone cortisol (or corticosterone in rodents), which is the primary glucocorticoid released from the adrenal glands during times of stress, and is easily measured in serum via standard laboratory tests. Cortisol can modulate a variety of biological effects in the body, namely, for the purposes of this review, overall immune function, and in cases of acute stress, can prime the immune system to prepare for the potential invasion of pathogens and subsequent infection.[7] Acting to suppress the APR during chronic stress, cortisol prevents excessive stimulation of the immune system by preventing the release of pro-inflammatory cytokines, decreasing B-cell Ab production and reducing inflammation, which could prove beneficial or detrimental to the animal, depending on a variety of factors. Release of glucocorticoids and catecholamines is considered a key component of the stress response, and the resulting increase in these stress hormones inhibits several pro-inflammatory cytokines (IL-6, IFN-γ and TNF-α).[13,14] Glucocorticoids also cause a shift from a primarily Th-1 effector-driven response to favor a Th-2-mediated humoral immune response[15] by inhibiting the production of, and responsiveness to, certain pro-inflammatory cytokines.[16,17]

Acute versus chronic stress

Acute stress occurs when an animal experiences a stressor for a short period of time, and is associated with the 'fight or flight' response and priming of the immune system, which promotes adaptation to a short-term stressor. Chronic stress, however, manifests when an animal experiences a prolonged insult to its homeostatic state, shifting the stress response from one that is preparatory to one that is suppressive across the entire immune system.[7] In contrast to chronic stress, during a response to an acute stressor an animal initiates restraining forces to prohibit an over-reaction from the central and peripheral components of the stress system. The anti-growth, anti-reproduction, catabolic and immunosuppressive effects of the acute stress response are intended to be of limited duration—temporarily beneficial to the individual. Prolonged subjection to stress and, subsequently, pro-longed secretion of glucocorticoids can lead to the development of pathological conditions. This shift from acute to chronic stress is dependent upon the animal and its perception of the stressor, the duration of the stressor and as its ability to overcome a stressful event based on previous exposure, genetics, gender, temperament and other contributing factors.[9,18]

Glucocorticoids and catecholamines have combined effects on monocyte/macrophage and dendritic cells to inhibit components of innate immunity and the APR, while promoting the production of Th-2 humoral-related cytokines, suggesting that stress-induced immune dysfunction primarily involves the innate immune system.[19] Recent research investigating the interaction between stress and immunity suggests that the APR produced by cattle in response to a pathogen is dependent upon natural variations associated with gender, breed and temperament.

Sexual dimorphism of the APR

Sexually dimorphic APRs have been demonstrated in human and animal models, suggesting that gender plays a role in an animal's ability to withstand infectious disease.[20–22] These differences are attributable to steroid hormones, specifically estrogens, androgens and progestins, that have immunomodulatory effects.[23,24] The specific role that different sex steroid hormones play in the APR is not completely clear; however, in vivo Ab production in human and animal models is enhanced by estrogen.[25,26] Conversely, androgens tend to suppress certain aspects of the immune response.[27]

Effects of estrogens on innate immunity

The female reproductive tract contains a full complement of immune cells involved in innate immunity. Yet, in order for reproductive functions such as spermatozoa or the developing fetus to thrive, local immunity within the uterus must be down-regulated to ensure survival of spermatozoa and the subsequent fetus. Therefore, increases in the female sex hormones estradiol and progesterone regulate the concentration and activity of leukocytes and other components of innate immunity in the reproductive tract throughout the reproductive cycle.[28,29] Changes in the production of natural anti-bacterial components, recruitment of phagocytic cells, including neutrophils, macrophages and dendritic cells, as well as changes in NK cell function and concentration occur throughout the estrous cycle. Regarding inflammation, estradiol is considered anti-inflammatory because it has been reported to suppress pro-inflammatory cytokine production and inflammatory cell recruitment.[30,31] Estradiol and progesterone also influence the APC functions of dendritic cells, and estradiol may influence dendritic cells to stimulate Th-2 responses, while simultaneously causing a decrease in production

of the Th-1 cytokines, TNF-α and IFN-γ.[32] Further evidence of sex-related regulation exists in the mouse model; female mice mount a more vigorous T-cell response and produce more Abs than males when immunized.[33,34] Kahl et al.[35] reported a greater APR after LPS challenge during estrus compared to diestrus in heifers, suggesting that the difference in the magnitude of pro-inflammatory response between phases of the estrous cycle may be due to the endocrine environment, particularly progesterone and estradiol concentrations. Further, existing evidence suggests that disease severity decreases during pregnancy, particularly during the third trimester when progesterone and estradiol concentrations are highest, while subsequent disease incidence is increased post-partum when these hormone concentrations are less.[36,37]

Effects of androgens on innate immunity

The endocrine system controls the development and expression of ornamentation, behavioral displays, and obligatory sperm production via testosterone and other adrenergic steroids in males.[38,39] In terms of mating success, high concentrations of testosterone represent libido; however, components of innate immunity, such as pro-inflammatory cytokines, are known to adversely affect male ornamentation, behavioral displays and sperm quality.[40,41] While testosterone has its reproductive benefits, it has also been shown to cause immune dysfunction.[42–44] Androgens may have stimulatory or suppressive effects on various components of the APR, depending on animal species. Testosterone suppresses immune cell differentiation and macrophage activation in mice and rats,[23] whereas production of IL-2 and IFN-γ in peripheral T-cells increased following castration of male rats, improving their ability to overcome viral and bacterial infection.[45] However, in hamsters, testosterone had a direct stimulatory action on immune

cells in vivo and in vitro, as evidenced by an increase in cell-mediated immunity.[46] Likewise, other studies suggest that immune function is positively correlated with increases in testosterone.[47,48] Kahl and Elsasser[49] reported greater serum concentrations of TNF-α and serum amyloid A in steers administered testosterone via implant (a growth promotant commonly used in beef production) following LPS challenge, but no difference in clinical signs were observed between steers administered testosterone and control animals. Interestingly, in a subsequent LPS challenge on the same animals, the TNF-a response was not different between control and testosterone-treated steers. Yet, there was a prolonged acute phase protein response in steers administered testosterone for a second time. Nevertheless, the effects of androgens on the APR in cattle are not as clear as the regulation of the APR by female sex hormones.

Sexual dimorphism of the APR in cattle

While the specific role that sex steroids may have in immune alteration in cattle is less clear, it is well understood that cortisol inhibits the APR and inflammation, as mentioned previously.[50,51] Carroll et al.[52] reported that recently weaned heifers tended to have greater cortisol concentrations than bull cohorts after LPS administration. Similarly, Henricks et al.[53] reported that Angus heifer calves had greater basal concentrations of cortisol than Angus bull calves. In contrast, purebred Brahman cattle administered LPS did not display sexual dimorphism regarding cortisol concentrations; however, systemic concentrations of TNF-α were greater in heifers than bulls.[22,54] Additionally, heifers displayed a greater and more sustained febrile response, more pronounced sickness scores, increased heart rate and increased neutrophil:lymphocyte ratio following an LPS challenge compared to bull calves.[55] In a similar study, Brahman heifers had greater basal

serum concentrations of cortisol, and subsequent to a corticotropin-releasing hormone (CRH) challenge, greater concentrations of IL-6, IFN-γ and TNF-α than bulls, suggesting that the APR in bulls may have been suppressed to a greater degree, either directly by CRH or indirectly by cortisol, than in heifers.[56] This further suggests that heifers may be less sensitive to the negative feedback effects of glucocorticoids and have an increased 'priming' of the immune system than bulls following acute stress.[57,58]

Variations in the APR based on gender are summarized in Table 1, and, based on these observations, incorporating different managerial practices for heifer versus bull calves during stressful periods (i.e. weaning, handling, feedlot induction) could be an important aspect of health management.

Table 1. Gender variations of the inflammatory response in cattle

Challenge	Heifers	Steers	Reference
LPS	greater cortisol	-	50
No challenge	greater basal conc. cortisol	-	60
LPS	greater heart rate, greater febrile response, greater sickness scores, greater neutrophil:lymphocyte ratio	-	68
CRH	greater basal conc. cortisol prior to challenge, greater IL-6, IFN-γ, TNF-α	-	54
LPS	greater TNF-α	-	53

Genetics and the APR

Genetics play an important role in shaping the physical and physiological characteristics of an animal, and studies have reported differences among breeds in multiple species. In cattle, Bos indicus and certain Bos taurus breeds, such as Senepol,

are more resistant to heat stress than breeds developed in Europe.[59,60] Different breeds of mice have shown variations in production of heat shock proteins and sensitivity to heat-induced neural-tube defects.[61] Henken et al.[62] observed differences in energy and protein metabolism among varying breeds of pigs, as evidenced by differences in growth rate and feed conversion. Therefore, differences in various characteristics, such as heat tolerance, growth traits and carcass traits are recognized. Perhaps less understood are variations in stress and the innate immune responses as a result of breed type.

Stress response variations attributable to breed

Research has shown that genetic differences exist associated with stress responsiveness. Differences have been reported for indices of the APR between Angus and Brahman Angus cattle when exposed to shipping stress, with Angus steers having greater total leukocytes and skin-test responses to phytohemagglutinin (PHA) than BrahmanAngus steers.[63] Similarly, Angus calves had greater total IgG immune response and IgM titers against pig red blood cells, and greater lymphocyte proliferation in response to PHA compared with Simmental calves,[64] which is indicative of a greater adaptive as a possible outcome of a greater APR and, therefore, stress response. Reed and McGlone[65] reported differences in neutrophil chemotaxis in two commercial lines of pigs when kept in an outdoor environment; however, no differences were observed between breeds when housed indoors. Greater post-stress adrenocorticotropic hormone (ACTH) concentrations after exposure to a novel environment were reported in Large White pigs when com-pared with Meishan pigs,[66] and while no immune parameters were evaluated, it is plausible that the immune responses of these breeds may have differed. In studies by Sutherland et al.,[67,68] which reported numerous breed effects on immune components, the

authors did not report any breed × stressor effects on immunity of pigs exposed to heat stress and crowding. Recent studies in cattle, however, have reported variations in the stress response between breeds. In these studies, physiological and blood serum differences were evaluated in heat-tolerant (Romosinuano) and heat-susceptible (Angus) Bos taurus cattle during a con-trolled heat challenge, with Angus cattle exhibiting greater respiration and sweat rates than Romosinuano cattle.[69] In a similar study observing the same two breeds (Romosinuano and Angus), Carroll et al.[70] reported greater cortisol responses inAngus cattle following LPS challenge, suggesting that Angus cattle more closely regulate the APR than Romosinuano cattle, given that cortisol serves as a potent anti-inflammatory hormone. These variations among breeds in response to stress and immune challenge may indicate a need to manage these breeds differently.

Breed effects on the APR

Genetics can also affect the APR in animals. Recent efforts have been made to select animals for greater disease resistance to improve health, performance and overall productivity, which requires characterization of immune parameters among varying breeds.[67] Genetic selection for Ab or adrenal responsiveness and its relationship to disease responsiveness has been implemented in mice,[71] poultry[72,73] and pigs.[74,75]

In pigs, breed effects have been reported to have an effect on immune traits and cortisol in response to restraint stress,[76] exposure to a novel environment[66] and bacterial challenge.[77] This is consistent with earlier research conducted by Rothschild et al.[78] and Meeker et al.[74] who reported breed differences in immune responses to different antigens in swine. Two commercial lines of pigs demonstrated differences in NK cell cytotoxicity and the lymphocyte proliferative response,[65] while, more recently, Sutherland et al.[67,68]

reported differences in neutrophil phagocytosis and NK cell cytotoxicity for different breeds of pigs.

Previous research in dairy cattle demonstrated a greater number of Ab plaque-forming cells in response to chick red blood cells developed in Brown Swiss calves than in Holstein-Friesian Aryshire and Guernsey calves.[79] Ballou[80] reported greater concentrations of TNF-α in Holstein calves compared with Jersey calves after challenge with LPS, suggesting that Jersey calves may be at a greater risk for morbidity during the post-weaning period. Angus cattle had a greater response to PHA during a skin-test than did Brahman × Angus crosses.[63] Additionally, Muggli et al.[81] reported differences in IgG concentration among Angus and Hereford cattle, and Engle et al.[64] observed greater immune responses in Angus cattle when compared with Simmental cattle. Piper et al.[82] reported differences in the expression of innate immune genes when investigating gene expression in the skin of Holstein-Friesian and Brahman tick-infested cattle. More recently, Carroll et al.[70] observed longer durations and greater concentrations of TNF-α and IL-1β in Romosinuano steers when compared with Angus steers following endotoxin challenge, suggesting that Romosinuano steers may have a more robust pro-inflammatory response than Angus cattle. Based on these studies, which are summarized in Table 2, it would be reasonable to conclude that management strategies may need to differ between various breeds dependent upon the challenges presented to these animals, and future research to investigate implementation of these management strategies in production settings is warranted.

Table 2. Breed variations of some immune and stress responses in cattle

Explanatory Variable	Breed Response	Reference
Chick red blood cells	greater number of Ab plaque-forming cells in Brown Swiss vs. Holstein-Fresian, Aryshire, and Guernsey	77
LPS[a]	greater conc. TNF-α in Holstein versus Jersey	78
PHA[b]	greater skin test response in Angus vs. Brahman x Angus	61
	greater IgG conc. in Angus vs. Hereford	79
PHA	greater IgG/IgM titers/lymphocyte proliferation in Angus vs. Simmental	62
Tick infestation	differences in skin gene expression between Holstein-Fresian and Brahman	80
LPS	greater durations/greater conc. TNF-α/IL-1β in Romosinuano vs. Angus	68
Shipping stress	greater total leukocytes in Angus vs. Brahman x Angus	61
Controlled heat challenge	Greater respiration/sweat rates in Angus vs. Romosinuano	67

[a] lipopolysaccharide
[b] phytohemagglutinin

Temperament and the APR

Definition of cattle temperament

Cattle temperament is defined as the reactivity to humans and novel environments,83 and is inherently linked to stress in that it involves an animal's response to physical and psycho-social stressors associated with livestock management procedures.[84–86] Temperament can be influenced by breed, gender, age and previous handling.[87–90] For example, Bos indicus and Bos indicus-crosses tend to be more high-strung or temperamental than Bos taurus cattle,[83,91] and steers are typically calmer than heifers.[92]

Additionally, heritability of exit velocity from a chute, and chute score, which are measurements of temperament, are affected by breed.[93,94] However, extensive handling early in life, and repeated exposure to the same stimuli over extended periods of time, can improve the temperament of cattle. Under the same conditions of transportation, lairage and management of slaughter, improving cattle temperament could potentially reduce the negative effects of temperament on carcass quality.[90,95,96] Social interactions among cattle can also affect behavior and temperament, especially if isolated rather than raised in a group.[97]

Effects of temperament on the stress response

Temperament can be described as animal's physical response to various stressors, and the stress response interacts with the APR; therefore, it is logical to speculate that differences in an animal's temperament would have an impact on the APR in some cases. In cattle and mice, secretion of various stress hormones have been positively linked to temperament,[98,99] and temperamental cattle tend to have greater basal concentrations of cortisol than calm cattle,[90,100,101] which may indicate that cortisol acts to protect temperamental cattle facing immune challenge by mitigating the APR and allowing the animal to more quickly return to a homeostatic state.

In mice, those showing high locomotion behaviors also had larger adrenals, greater concentrations of glucocorticoids and lesser concentrations of TNF-α following tail nicking, indicating greater hypothalamic–pituitary– adrenal (HPA) axis activation and, subsequently, a suppressed pro-inflammatory response.[102] Curley et al.[98] observed greater basal concentrations of cortisol in temperamental heifers; however, following ACTH and CRH challenge, temperamental cattle had a lesser response than calm cattle,

demonstrating that character-istics of the stress response vary with temperament. When challenged with LPS, temperamental Brahman bulls had lower rectal temperature, less sickness behavior and failed to produce an epinephrine response, though it is not completely clear whether this is beneficial or detrimental to the health of temperamental animals.[99] The lesser sickness behaviors, lower rectal temperature responses and greater basal cortisol concentrations appear to contradict previous assertions that a greater or more robust APR is beneficial to the health of an animal. It still remains unclear how temperamental cattle appear to be less affected by an inflammatory challenge; however, it has been hypothesized that this is owing to differences in energy utilization.[103]

Additionally, as temperamental cattle produce a blunted response to HPA axis activation it is possible that the greater circulating cortisol concentrations observed in temperamental cattle alter the cytokine response during inflammation. Further research is necessary in order to fully understand the influence of temperament on the APR following endotoxin exposure.

Effects of temperament on the immune response

Adaptive or humoral immunity, which is activated by the APR, is also affected by temperament and may suggest variations in the innate immune response of temperamental animals. Vaccination, or rather immunization, is an important aspect of the immune response. It has been reported that temperamental calves have a reduced Ab response to vaccination when compared with calm calves that may be due to higher circulating concentrations of glucocorticoids, which inhibit the immune response. Lower in vitro lymphocyte proliferation and lower in vivo vaccine-specific IgG concentrations were observed in temperamental steers when compared to calm steers.[104] In another

study, lower serum concentrations of IgG, as well as a diminished ability of isolated lymphocytes to produce IgM and proliferate, was reported in temperamental cattle.[105] Determining whether or not the differences in the innate immune response of temperamental cattle is beneficial or detrimental to the long-term health of those animals has not been fully elucidated; however, it is clear that there are variations in immune function due to temperament (summarized in Table 3) that warrant more applied studies in order to determine potential changes in the management of these animals for optimal production.

Table 3. Variations of stress and immune responses due to temperament in cattle

Explanatory Variable	Temperamental	Calm	Reference
Vaccination	reduced Ab response	-	102
Exit velocity	greater basal conc. cortisol	-	88
LPS	greater basal conc. cortisol; no epinephrine response	-	98
Response to complex novel environment	diminished ability of lymphocytes to produce IgM	greater RT[a]/SBS[b]	100
		greater IgG	103

[a] rectal temperature
[b] sickness behavior score

Conclusions

The APR involves the production of pro-inflammatory cytokines and acute phase proteins, increases in body temperature and behavioral changes, and is critical to an animal's maintenance of homeostasis. A homeostatic balance is necessary for optimal livestock production, where growth of the animal is a priority. Natural variations in the APR due to gender, breed and temperament are well documented. These natural vari-

ations also serve as modulators of the stress response, which underlies a different impact of stress on innate immune function. Understanding that cattle have different immunological responses, based on naturally occurring variations such as these, may be the foundation of new studies on how to effectively manage cattle so that health is optimized and production is benefited.

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