STUDIES OF IMMUNOGENETIC VARIATION IN CATTLE

A Dissertation

by

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ABSTRACT

Genetic selection for animal health and disease resistance has been limited, likely due to the challenges of performing controlled studies with an industry relevant phenotype. Studies in large populations of animals of unknown relationship pose challenges for genome wide association studies of disease resistance. The aims of this project were to characterize variation in the bovine major histocompatibility complex (BoLA), a specific region of the bovine genome known be critical for development of immune response, and then investigate individual variation in host immunity to a specific viral pathogen of cattle, bovine viral diarrhea virus (BVDV).

Cattle “homozygous” for BoLA were identified from approximately 2,000 head of Holstein calves in large genome wide association studies. Cattle were genotyped on the Illumina BovineHD SNP chip and PHASED for the characterization of BoLA haplotypes. Among 160 “homozygous” animals, we identified 38 different haplotype groups. The 38 haplotype groups maintained the structure predicted by earlier studies that identified 50K SNP haplotypes, but demonstrated that more diversity is present among these 38 BoLA haplotype groups than was indicated by the 50K haplotypes. Among the 1,221 SNPs genotyped on the HD chip were 230 SNPs with no calls in at least one of the 160 homozygous animals. The no call SNPs are located predominately in regions predicted to contain copy number variation, and no call SNPs appear to likely mark regions of polymorphic structural variation otherwise undetected in the SNP

defined haplotypes. This structural variation may be important for future genome association studies.

Cattle diseases are often difficult to diagnose due to presentation with different disease phenotypes, ranging from subclinical to lethal. Likewise, immune response to vaccination is also variable and may be related to individual differences in disease susceptibilities. To evaluate individualized response to BVDV vaccination, we evaluated protection afforded by commercial vaccines against a BVDV challenge. The results from the BVDV challenge study indicate that measuring antibody titer as a response to BVDV vaccination may not be predictive of a protective immune response. Rectal temperature alone for health classification missed up to 50% of animals with subclinical disease.

Variation in host immunity appears to underlie the response to pathogens and likely to vaccination as well. Host differences in immunity between *Bos indicus* and *Bos taurus* cattle were evaluated subsequent to BVDV vaccination. Differences in baseline immune cell counts were observed. Indicine cattle had higher white blood cell counts primarily influenced by the 2-fold higher neutrophil levels. Response to vaccination was primarily observed as an innate immune response with an increase in neutrophils. The largest change was in neutrophil response observed in the taurine calves. Immunosuppression from the modified live vaccination was greater in the indicine calves compared to taurine calves. However, a combination of vaccination protocols appear to mitigate the immunosuppression observed in the indicine cattle.

DEDICATION

This work is dedicated to three very essential people in my life: my mom and dad, Anne and Dale Downey, and my grandmother, Margret Downey. Throughout my life they have provided the building blocks that make me who I am and have given me the strength and perseverance to complete my Ph.D. Their constant support, dedication, and faith in me during this process and throughout my life has enabled me to be here.

To my parents, words will never express my gratitude for all you have done for me. The life lessons of commitment, responsibility, and hard work; the passion for the livestock industry; and the development of my moral character have all shaped me into who I am today. Thank you for your patience, therapy sessions, and encouragement that have kept me going all these years. You have shown me that with perseverance, faith and a little bit of stubbornness I can achieve anything I choose.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Animal agriculture currently faces many challenges related to animal health and disease (WAGNER 2000; SJEKLOCHA 2013; SMITH 2013; REED 2015) as the industry is forced to find alternatives to antibiotics, both feed grade and over-the-counter antibiotics for treatment (SJEKLOCHA 2013; BELKNAP 2015). Animal health and disease is an issue that impacts production, product quality, and the public perception of the livestock industries (WAGNER 2000; SMITH 2013; JAYNES 2015). The annual cost of disease to the beef industry is nearly impossible to estimate but is thought to range in the multi-billions of dollars annually. A single disease, Bovine respiratory disease (BRD), has been estimated to cost the U.S. cattle industry from 750 million dollars to upwards of 3 billion dollars annually (GRIFFIN 1997; SNOWDER *et al.* 2006; HOLLAND *et al.* 2010; VARGA 2015). Brucellosis cost the industry an estimated 400 million dollars in the early 1950s, but with implementation of eradication programs the estimated annual cost to the industry is now less than 1 million dollars (SELEEM *et al.* 2010). The annual economic loss to Johne's disease in cattle is estimated at 200 to 250 million dollars (OTT *et al.* 1999).

Public perception of disease outbreaks can devastate an industry and cost millions of dollars as was experienced with the bovine spongiform encephalopathy (BSE) outbreak. The 2004 outbreak of BSE has been estimated to cost the U.S. 3.2 to 4.7 billion dollars through loss of export markets and recalls on retail products (PENDELL *et al.* 2007).

Many of the challenges facing the beef industry can be addressed by cattle genetics (WAGNER 2000), but the role(s) of genetics in these complex phenotypes remains to be determined. Decisions about genetic selection are made in the cow-calf sector of the industry (WAGNER 2000), yet many of the major disease and health issues facing the industry occur in other sectors of the beef industry. Improving our understanding of the mechanisms that control disease susceptibility in cattle can enhance the use of genetic selection to improve animal health and to develop more effective preventatives, such as vaccination, for benefits at all levels of cattle production.

The focus of the research presented in this dissertation was two-fold: (1) to elucidate genetic variation in the bovine major histocompatibility complex (MHC) that may underlie many disease and immune response phenotypes and (2) to evaluate variation in host immunity to commercial vaccinations and subsequent viral challenges that model respiratory disease. Genomic association studies with complex traits present with many challenges. In a recent genomic association study for bovine respiratory disease complex, the genetic association analysis did not reveal associations of clinical score with the MHC (NEIBERGS *et al.* 2014). This result is somewhat surprising as variation in the MHC is frequently associated with a variety of disease susceptibilities and immune phenotypes. The first goal was to investigate the ability of SNPs present in BoLA on the BovineHD SNP chip to capture variation among diverse BoLA haplotypes. The second half of the dissertation is focused on characterization of immune response and vaccine protection, which could be used to identify the genetic signatures that are predictive of

key pathways for immune protection (FURMAN AND DAVIS 2015). The second goal was a field trial to evaluate protection from commercial vaccinations against a bovine viral diarrhea virus (BVDV) challenge and to determine if current metrics for diagnostics of viral infections can identify diseased animals. The third goal was to compare variation in host immune responses between *Bos taurus indicus* and *Bos taurus taurus* calves vaccinated with commercial BVDV vaccines.

Genetics of Cattle

Cattle (bovines) have been used by humans for thousands of years as draft animals, for their resources (hides and fertilizer), and primarily as food sources of milk, blood, and meat (ELLIS AND HAMMOND 2014). Bovines are even-toed ungulates that belong to the order Cetartiodactyla (BURT 2009). Members of the Cetartiodactyla are estimated to have appeared nearly 60 million years ago, and domesticated cattle likely diverged from a common ancestor 250 thousand years ago (BURT 2009; CONSORTIUM 2009). Two extant sub-species, *Bos taurus taurus* and *Bos taurus indicus,* originated from the common ancestral aurochs, *Bos taurus primigenius* (BURT 2009; CONSORTIUM 2009; PORTO-NETO *et al.* 2013; DECKER *et al.* 2014). The two sub-species originally occupied geographically divergent regions, with indicine cattle found in the Indus Valley of India and taurine cattle in the Fertile Crescent of what is now Iraq (CONSORTIUM 2009; PORTO-NETO *et al.* 2013). A second domestication occurred within taurine cattle to produce the African taurine cattle (MCTAVISH *et al.* 2013; DECKER *et al.* 2014). Since the initial domestications, cattle derived from the two sub-species have undergone

various selection pressures to enhance specific production traits and generate almost 800 different cattle breeds (BURT 2009; VILLA-ANGULO *et al.* 2009; PORTO-NETO *et al.* 2013). Cattle breeds have been highly influenced by the challenges of the geographical regions of origin, as indicine cattle are notably known for drought and heat tolerance (MCTAVISH *et al.* 2013; RAMEY *et al.* 2013; DECKER *et al.* 2014).

The phenotypic diversity observed among contemporary breeds of cattle come from unique evolutionary histories and geographical dispersal and isolation (DECKER *et al.* 2014), leading to unique combinations of genes among breeds (BLOTT *et al.* 1998; RAMEY *et al.* 2013). The development of recognized breeds and the presence of strong artificial selection within breeds has caused some concern for preservation of original genetic diversity present in the ancestral aurochs (RAMEY *et al.* 2013). Conflicting reports exist in the scientific literature as to which of the contemporary bovine sub-species is more genetically diverse (CONSORTIUM 2009; PORTO-NETO *et al.* 2013). This controversy may arise from breed biases on the SNP chips used to assess diversity, as SNPs have been selected from two predominate breeds, Angus and Holstein, and two Hereford reference sequences (RAMEY *et al.* 2013; DECKER *et al.* 2014), all of which are taurine breeds. Whether improvements in production traits will continue as genetic variation is reduced is a growing concern (VILLA-ANGULO *et al.* 2009).

Genome wide association studies (GWAS) for improved feed efficiency, reproduction, growth and performance, and other traits are providing tools for enhanced selection for

improvement in quantitative traits (SAATCHI *et al.* 2014). Breed differences, especially differences between indicine and taurine breeds, affect stress response as well as the acute immune response (HUGHES *et al.* 2014). Other reported breed differences include susceptibility to BRD, where cross-bred calves have an advantage over purebreds (SNOWDER *et al.* 2006). Indicine breeds have the largest advantage with reduced incidence rates for BRD, resistance to ticks and parasites (MORAN *et al.* 1996; CUSACK *et al.* 2007). Little to no selection pressure has been implemented for animal health or disease resistance and susceptibility, primarily due to the lack of genetic-associations for selection in population studies. This may be partially due to the complexity of phenotypes, complications and expense of collecting quality data, and limitations to understanding the genetic mechanisms that underlie immune response and disease resistance.

Results of GWAS for complex production traits like feed efficiency are population dependent and the identified SNPs often account for only a small proportion of the phenotypic variance (SAATCHI *et al.* 2014). Similar observations have been published for disease studies (SNOWDER *et al.* 2006; NEIBERGS *et al.* 2011; VAN EENENNAAM *et al.* 2014). Some genetic regions identified in disease resistance/susceptibility were based on segregation in pedigreed families (UNTALAN *et al.* 2007) but such results are difficult to replicate in studies of unrelated populations (NEIBERGS *et al.* 2014; CASAS *et al.* 2015). One of these regions is the bovine major histocompatibility complex also known as the Bovine Leucocyte Antigen (BoLA) complex. GWAS is used to directly identify

genomic variants that are associated with phenotypic traits, especially those that are difficult and costly to measure. Gene markers have been associated with a number of traits, yet the region tagged by any one of these markers accounts for a very small proportion of the total phenotypic variance, suggesting that undiscovered variants account for the remaining phenotypic variance or that the traits are controlled by many more QTLs than originally thought (MANOLIO *et al.* 2009; SADEE 2012; ZAITLEN AND KRAFT 2012; ZUK *et al.* 2012; VINKHUYZEN *et al.* 2013; MONTE AND VONDRISKA 2014; SADEE *et al.* 2014). SNPs alone do not appear to be able to identify all of the genomic variation underling complex phenotypes, suggesting that gene-gene interactions or structural variants unmarked by SNPs may be responsible for some of the missing variance (ZUK *et al.* 2012).

Major Histocompatibility Complex Structure

The major histocompatibility complex (MHC) was first described in mice (GORER AND SCHUTZE 1938; SNELL 1948). It has since been described in many vertebrates and nearly all mammals in some detail (KELLEY *et al.* 2005; KAUFMAN 2011). The bovine MHC was described in the late 1970's (AMORENA AND STONE 1978; SPOONER 1978) and was later renamed the bovine leukocyte antigen, *BoLA* (SPOONER 1979). The BoLA complex is a gene-dense region located on bovine chromosome 23, and composed primarily of genes associated with immune response or disease susceptibility (TAKESHIMA AND AIDA 2006; MIYASAKA *et al.* 2011). BoLA is divided into three regions, classes I, II, and III, based on gene content and displays remarkable conserved synteny with the MHCs of

other mammalian species (KELLEY *et al.* 2005), as illustrated in Figure 1.1. The organization of BoLA differs from that of other mammals at the class II region. The BoLA class II region has apparently been disrupted by a large inversion to place a portion of the class II region, called IIb, near the centromere, separated from the main MHC region by approximately 20 Mb of non-MHC DNA (CHILDERS *et al.* 2006). The BoLA class IIa region contains the functionally expressed DQ and DR genes and is tightly linked to the class II and class I regions, creating a diverse cohort of MHC haplotypes composed of more than 230 translatable genes (DAVIES *et al.* 1997). The classical genes of BoLA class I and IIa are under diversifying selection and are the most polymorphic genes in mammalian genomes. A sizable portion of variation in BoLA class I and IIa regions has been attributed to deletions and duplications, CNV and segmental duplications, resulting in a number of different gene configurations (ELLIS *et al.* 1999; BABIUK *et al.* 2007; HOU *et al.* 2012a; SCHWARTZ AND HAMMOND 2015). Different gene configurations may result in varied immune responses that have not previously been detected by SNPs.

Class IIa receptors are encoded by four pairs of genes: DQα and β, DRα and β, DNα and β, and DOα and β, (BECK AND TROWSDALE 1999; KELLEY *et al.* 2005; BAKER *et al.* 2006). Class I and IIa genes have been the focus to understand the immune function of the MHC. MHC class IIa region, composed of functionally expressed DQ and DR genes, is tightly linked to the class I region creating a diverse cohort of MHC haplotypes (DAVIES *et al.* 1997). MHC class II alleles are redundant and highly polymorphic,

enhancing the repertoire of epitopes that an individual can recognize (NORIMINE AND BROWN 2005). Class II DQα and β and DRβ are the most polymorphic genes in the bovine MHC, similar to other species (ANDERSSON *et al.* 1986). Five different DQα and β loci and three DRβ loci have been identified, though the number of DQα loci is somewhat controversial (ANDERSSON *et al.* 1988; BALLINGALL *et al.* 1998; GELHAUS *et al.* 1999b). Most haplotypes express two DQα and β loci and one DRβ functionally expressed gene, the number of DQ genes is shown to vary with haplotype (ANDERSSON *et al.* 1988; GELHAUS *et al.* 1999a; GELHAUS *et al.* 1999b; TAKESHIMA AND AIDA 2006). To date, 106 DRβ3, 46 DQα, and 52 DQβ alleles have been reported (NORIMINE AND BROWN 2005; BAXTER *et al.* 2009). Bovine MHC class I is composed of at least 10 classical and non-classical genes and pseudogenes; four of these genes are transcribed but their expression is highly variable among individuals (BABIUK *et al.* 2007). The variation in the classical class I genes expressed in bovine are greater than those reported in other species (ELLIS AND HAMMOND 2014). At least 120 distinct class I sequences have now been identified and deposited into the Immuno Polymorphism Database for cattle (http://ebi.ac.uk/ipd/mhc/) (TAKESHIMA AND AIDA 2006; ROBINSON *et al.* 2010; ELLIS AND HAMMOND 2014). Allelic polymorphisms are associated with the antigen-binding region which is used to define the specificity of the acquired immune response and for haplotype identification (BALLINGALL *et al.* 1998). Polymorphic MHC genes and variation in the MHC haplotypes contributes to the diverse range of immune responses.

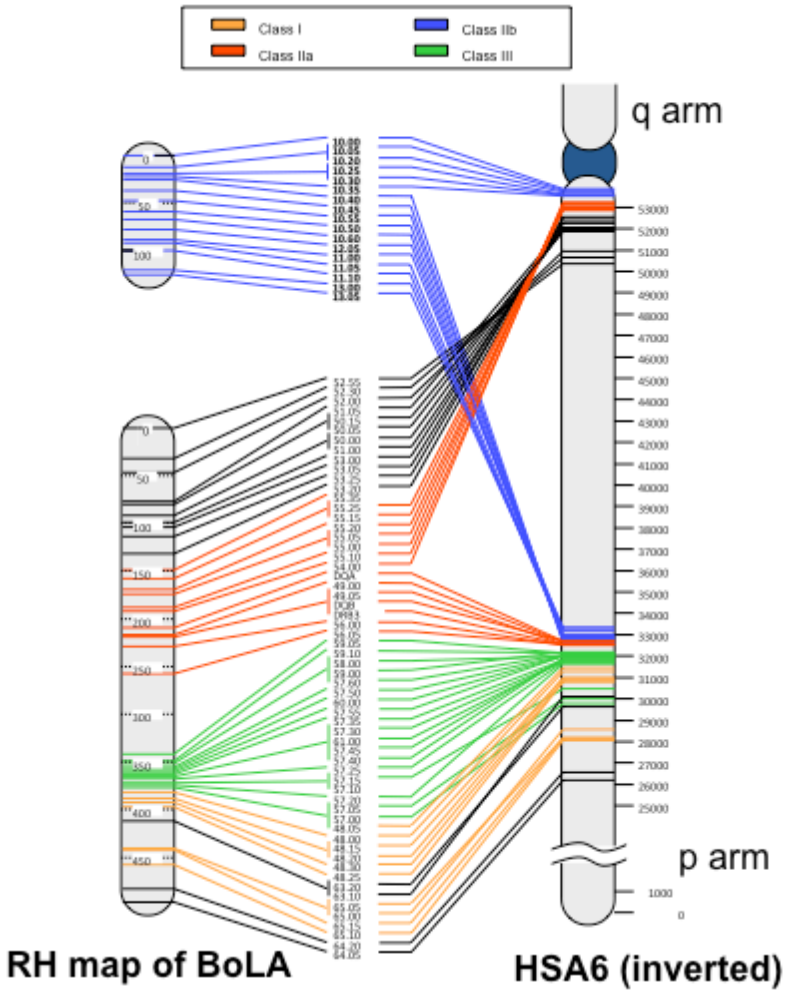


Figure 1.1 Comparative Map of BoLA from Radiation Hybrid Panel aligned to HLA. Figure from (BRINKMEYER-LANGFORD et al. 2009).

Polymorphisms

The MHC contains some of the most polymorphic genes in mammalian genomes. High levels of polymorphisms expressed in the antigen presenting genes of the MHC contribute to the diverse immune responses developed to host pathogens and individual variation of expressed immune response (BABIUK *et al.* 2007). Polymorphisms in BoLA class I and II genes influence the adaptive immune response through peptide processing, transport and binding, antigen presentation, and cytokine networks to play a central role in the development of an effective immune response (TAKESHIMA AND AIDA 2006; MIYASAKA *et al.* 2011). SNPs associated with vaccine immunity and disease susceptibility in the HLA class II genes determine the specificity of the immune response and play a role in conferring disease susceptibility (ORANGE *et al.* 2011; MCKINNEY *et al.* 2013; WU *et al.* 2013). Similarly, SNPs and insertion/deletion polymorphisms have been associated with variation in individual cattle (SCHRIDER AND HAHN 2010). The frequencies of polymorphic alleles differ among breeds and might account for breed differences in the specificity, intensity or duration of the immune response, producing a diversity of immune phenotypes (BAXTER *et al.* 2009; MIYASAKA *et al.* 2011).

Haplotype Structure

The haplotype structure across the MHC is conserved in most mammals, however cattle and other ruminants are unique in the expression of multiple DQα and DQβ loci, which may contribute to the variation in the immune phenotypes (SCOTT *et al.* 1987;

ANDERSSON *et al.* 1988). At least four DQβ loci have been identified in cattle, and strong evidence exists for a fifth DQβ loci, but only two DQα and DQβ loci have been identified in any single haplotype (GELHAUS *et al.* 1999a; GELHAUS *et al.* 1999b; SCHWARTZ AND HAMMOND 2015). In haplotypes with single and duplicated versions of DQ loci, all genes appear to be functional (GELHAUS *et al.* 1999a; GELHAUS *et al.* 1999b). The presence of a heterozygous DQ duplication may lead to increased diversity of immune phenotypes, and may explain some of the variation not previously identified by SNP-haplotypes. To support this idea, Gelhaus et al. suggested that in the polypeptides encoded by DQα5 and DQβ5 together in the same haplotype are able to form ”hybrid” DQ receptors with divergent immunological functions (GELHAUS *et al.* 1999b). The presence of a duplication of DQ, increases the potential to amplify class II molecules expressed at the cell surface to produce inter- and intra-haplotype pairing of the alpha and beta chains (GLASS *et al.* 2000). Duplications of genes within given haplotypes might offer an advantage to the variation of the immune response mounted in cattle. In addition to the diversity of the DQ genes in BoLA class II region, there are six known class I classical MHC loci in bovine (ELLIS AND HAMMOND 2014; SCHWARTZ AND HAMMOND 2015). Between one and three class I genes are expressed in a haplotype, and the expression is not consistent from animal to animal (PARHAM 1999; TAKESHIMA AND AIDA 2006).

Approximately 80 BoLA haplotypes have been identified using the 50K SNP genotyping platform in a diverse cohort of cattle breeds, primarily of taurine lineage (FRITZ 2009).

Individuals can have identical genotypes yet contain variants that are not identified by genotypes, such as sequence to sequence variation or possible copy number influences on gene expression, that allow for varied phenotypic specificities (USINGER *et al.* 1981). More than one copy of a gene may be expressed in some haplotypes and be unaccounted for by current SNP-based haplotype identification. Unmarked variation in copy number and gene expression might influence diversity in immune responses that are associated with a single haplotype (ELLIS *et al.* 1999). Absolute gene numbers have not yet been captured in the current haplotype identification system and polymorphisms in these genes may drive the variation that underlies association of BoLA haplotypes with immune phenotypes.

Haplotype structure is more conserved between breeds within a subspecies group as opposed to breeds between subspecies groups (VILLA-ANGULO *et al.* 2009). However re-sequencing of BoLA haplotypes to a depth sufficient for de novo assembly may be required to fully characterize the variation in the bovine MHC and document variation in gene content within haplotypes (BIRCH *et al.* 2006). Variation in BoLA class I haplotypes may indicate more variation is present than what has been captured by SNP-defined haplotypes.

Homozygous Animals

Using DNA samples from individuals homozygous for the MHC greatly simplifies the studies of this highly complex region of the genome by removing the confounding

effects presented by high levels of polymorphisms and heterozygosity. This approach has been used in a number of studies in humans and cattle (COLLEN *et al.* 2002; NORMAN *et al.* 2015). Not only are samples from MHC homozygous individuals advantageous for understanding BoLA haplotypes, but they also simplify immunological studies. BoLA homozygous animals enable MHC allelic associations with epitope determinants to be studied (COLLEN *et al.* 2002). Identification of MHC alleles and haplotypes that display the largest repertoire of pathogen-derived epitopes, as well as those alleles that encode receptors that efficiently present antigens from the most prevalent strains of pathogens will optimize host immune response. In a study of homozygous animals, variation in the number of recognized epitopes was observed among individuals, but the immune response by animals within a DRβ3* haplotype was consistent (COLLEN *et al.* 2002). This suggest that the DRβ3* locus and haplotypes are important for the recognized haplotypes, but there are alternative genetic components that can affect the robustness of the epitope response. Understanding the MHC haplotypes that respond to an increased number of epitopes could serve as a genetic selection program for animals that have increased immunity to viral pathogens and strains for increased disease tolerance. In addition, identification of the most commonly recognized epitopes by individuals could be used to develop new vaccines and improve vaccine efficacy.

Structural Variation in the Genome

The genome structure is constantly undergoing changes and rearrangements (ZHANG *et al.* 2009a; STANKIEWICZ AND LUPSKI 2010), however the phenotypic consequences of many structural changes are unknown. Genomic structural variation includes: CNVs, segmentation duplications, small insertions, duplications and deletions, inversions and translocations (FADISTA *et al.* 2010; STANKIEWICZ AND LUPSKI 2010). SNPs were originally thought to be the major source of individual phenotypic variation, but undefined structural variation contribute significantly to phenotypic diversity (REDON *et al.* 2006; FADISTA *et al.* 2010). CNVs account for more total sequence than SNPs and are large enough to encompass whole genes and sets of genes. Therefore, CNVs have a potential for more significant effects on evolution, fitness, and genetic diversity (REDON *et al.* 2006; ZHANG *et al.* 2009b; FADISTA *et al.* 2010; SCHRIDER AND HAHN 2010; HOU *et al.* 2012b). Characterization of genetic variation in livestock species is an important step towards linking genes or genomic regions with phenotypes (STOTHARD *et al.* 2011). Detection of CNVs in the immune specified region of the genome might explain variation in immune response.

Copy Number Variation

Redon et al. (2006) defined a CNV as 1 Kb or larger, however other studies have identified CNVs of smaller sizes (ZHANG *et al.* 2009a; CONRAD *et al.* 2010; DOAN *et al.* 2012). Identified CNVs have included translatable genes, functional elements, and noncoding RNAs; many of which have not been associated with phenotypes (REDON *et*

al. 2006). The GC content associated with CNV regions has been shown to be slightly higher than the GC content of the whole genome, suggesting CNVs arise more frequently in gene-rich regions (FADISTA *et al.* 2010). The CNV detection resolution depends on the design of the array. This presents a challenge not only to detect CNVs but also to characterize expression and associate CNV changes with phenotypes. Now that arrays with higher resolution have been designed, smaller CNVs have been reported to be as frequent as large (>1 Kb) CNVs (FADISTA *et al.* 2010). Due to the design of SNP arrays, the smaller CNVs would not have likely been detected and therefore may not be accounted for in haplotype characterization.

CNVs account for a significant source of variation in mammals (FADISTA *et al.* 2010). CNVs are associated with quantitative phenotypes and to be known causative for genetic disorders (FADISTA *et al.* 2010). However, little is known about CNV variation in relation to phenotypic diversity in the bovine immune response. The proportion of the genome with predicted CNVs varies between 2.3 and 4.2% among individual humans (TENNESSEN *et al.* 2012), suggesting that individual variation may be associated with differences in immune response (SCHRIDER AND HAHN 2010; HOU *et al.* 2012b; TENNESSEN *et al.* 2012). Pairwise comparisons of taurine and indicine cattle suggested that CNV differences between subspecies are greater than across breeds within a subspecies (BICKHART *et al.* 2012). Stothard *et al.* (2001) have shown that genomic regions enriched with CNVs are breed dependent, which may reveal regions under selection pressure. Breed specific CNVs may also be relevant to the unexplained

haplotype variation that exists between breeds. Disease resistance may be influencing selection pressure for the enriched immune function genes present in CNVs. CNV detection and variation may help interpret diverse expressed immune responses that have previously been associated with identical haplotypes.

Association with Disease

Variation in the MHC has been associated with individual differences in immune response and disease susceptibility in many species. At least 100 different diseases in humans have been associated with HLA (KULSKI *et al.* 2002; KELLEY *et al.* 2005). BoLA association may be more dependent on the type of immune response phenotype (humoral versus cell mediated or innate versus adaptive) used for the association analyses. Diversity of the MHC class I region may be more likely associated with intracellular immune response and disease resistance, while the class II region may be of increased importance for extracellular pathogen phenotypes and humoral immunity responses (ELLIS AND HAMMOND 2014). Disease association studies in cattle have shown large variability in BoLA genes associated with immune response. In cattle, alleles of class II are associated with animal-to-animal variation in susceptibility to hoof-and-mouth disease, dematophilosis, mastitis, bovine leukemia virus, and tick resistance (LEWIN AND BERNOCO 1986; XU *et al.* 1993; DIETZ *et al.* 1997a; DIETZ *et al.* 1997b; SHARIF *et al.* 1998; UNTALAN *et al.* 2007; FADISTA *et al.* 2010; MIYASAKA *et al.* 2011). An indirect relationship between health and production traits was shown to depend upon which DRβ3 allele was present for mastitis resistance (OPRZADEK *et al.* 2012). BoLA

class II alleles DRβ3*2002 and DRβ3*0701 have been associated with BVDV-specific epitope responses in $CD4^+$ T-cells (COLLEN *et al.* 2002). In some diseases, affected individuals share a common haplotype at a higher frequency than would be expected by chance, suggesting a haplotype association with disease susceptibility (TODD *et al.* 1988). BoLA heterozygotes have an advantage of enhanced resistance and increased diversity of antigens presented and recognized (TAKESHIMA *et al.* 2008). Genetic variants of IFN-γ gene were reported to affect tick resistance in cattle (MARYAM *et al.* 2012).

Gene-by-Gene Interactions

SNP-based genetic association tests primarily seek single SNP genetic association; however complex traits are not likely controlled by a single gene or even a few genes. In many species, including both humans and bovine, immune related paralogous gene regions are located on at least 3 other chromosomes in addition to the MHC (PARHAM 1999). Evidence has been shown in human for an interaction between killer inhibitor receptors (KIR) and MHC haplotypes and associations with disease and immune regulation of viral infections (CAMPILLO *et al.* 2013; CARRILLO-BUSTAMANTE *et al.* 2013; NEMAT-GORGANI *et al.* 2014). Similarly, cattle natural killer cell diversity is influenced by the MHC, indicating interaction between the natural killer receptors (KIR and killer cell lectin-like receptors (KLR)) with MHC class I haplotypes (ALLAN *et al.* 2015). The interactions between KIR, KLR and the MHC class I haplotypes were reported to be independent of individual variation (ALLAN *et al.* 2015), suggesting that

epistatic variant interactions might not only account for unexplained variation but might also identify associations in phenotypes with large individual variation. Studying the interaction of immune related genomic variants may identify more variance associated with complex disease traits (ZUK *et al.* 2012).

Bovine Respiratory Disease

The second major objective of my dissertation research is the investigation of the variability in immune response of individual cattle exposed to BVDV and receiving BVDV vaccinations. Bovine respiratory disease (BRD) is the leading cause of mortality in cattle feedlots responsible for the single largest financial loss in the U.S. cattle industry for more than 20 years (SNOWDER *et al.* 2006; VARGA 2015). BRD is present in 97% of large feedlots in the U.S. (DUFF AND GALYEAN 2007a), making it one of the most important diseases facing the U.S. beef industry, with an estimated annual economic impact of 750 million to 3 billion dollars (SNOWDER *et al.* 2006; DUFF AND GALYEAN 2007a; RICHESON *et al.* 2013b; VARGA 2015). Approximately 24.3% of weaned and mature cattle die from BRD, and 28.7% of all cattle deaths are associated with BRD (National Agriculture Statistics Service 2006). BRD accounts for 60-85% of the morbidity and up to 45-60% of mortality in feedlot cattle (FULTON *et al.* 2002; SNOWDER *et al.* 2006; RICHESON *et al.* 2013b; VARGA 2015).

BRD is a multifactorial disease influenced by environmental effects and stressors, host response, and various bacterial and viral pathogens (DUFF AND GALYEAN 2007a;

SRIKUMARAN *et al.* 2007; MOSIER 2014). Stresses related to management, such as comingling, transportation, and processing of cattle, are associated with increased risks for BRD in calves (DUFF AND GALYEAN 2007a; RICHESON *et al.* 2013a; MOSIER 2014). Chronic stress, defined as stress that persists for days to weeks or longer (DHABHAR 2008), suppresses the immune response (DHABHAR 2009; HUGHES *et al.* 2014) and detrimentally affects performance (CARROLL AND BURDICK SANCHEZ 2013). Stress can affect the host immune response and can increase disease if cattle are chronically stressed at critical times (HUGHES *et al.* 2014). High stress periods associated with feedlot operations often occur simultaneously with exposure to pathogens or vaccination, potentially suppressing the immune response.

A causative pathogen associated with BRD has yet to be identified, due in part to a variety of pathogens detected in individual BRD cases (KLIMA *et al.* 2014). Multiple pathogens are often isolated during analysis of BRD cases (FULTON *et al.* 2006), complicating the identification of causative pathogens and the understanding of host-pathogen interactions. Analysis of BRD lung and nasopharynx samples has shown that as many as 97% of cases have multiple candidate pathogens present at clinical diagnosis, suggesting that identification of a single pathogen associated with BRD may be an unrealistic objective (KLIMA *et al.* 2014). BRD has been associated with 5 or more viral and 3 or more bacterial pathogens. Viral pathogens identified as possible causative agents of BRD include: bovine herpesvirus-1, bovine respiratory syncytial virus, bovine viral diarrhea virus, parainfluenza-3 virus, and possibly bovine coronavirus (FULTON *et*

al. 2002; SRIKUMARAN *et al.* 2007; MOSIER 2014). Candidate bacterial pathogens associated with BRD include: *Manheimia haemolytica*, *Pasturella multocida*, *Histophilus somni*, and *Mycoplasma bovis* (SRIKUMARAN *et al.* 2007; MOSIER 2014). These viral and bacterial pathogens are also found in unaffected, healthy animals, suggesting that pathology resulting from exposure to pathogens or increased pathogen burden may be secondary and a consequence of some other predisposing factor(s). The most common viral pathogen found associated with bacterial pathogens in feedlot BRD is bovine viral diarrhea virus (BVDV) and BVDV exposure may be an early predisposing event in BRD (FULTON *et al.* 2002; KLIMA *et al.* 2014).

Another challenge in the diagnosis and study of BRD is the range of clinical phenotypes observed among infected animals, the presence of subclinical phenotypes, and the excessive cost of well-defined phenotypes in animals for research. BRD is associated with changes in blood leukocyte profiles, in red blood cells and eosinophils (RICHESON *et al.* 2013b). The depletion of CD4 and CD8 T-cells by BVDV infections may create high at-risk individuals due to immunosuppression from the BVDV infection (RHODES *et al.* 1999; COLLEN AND MORRISON 2000; SRIKUMARAN *et al.* 2007).

Bovine Viral Diarrhea Virus (BVDV)

Bovine viral diarrhea virus (BVDV) is a positive-sense single-stranded RNA virus with a genome of ~12.5 KB and is a pestivirus of the *Flaviridae* family, first described in 1946 (RIDPATH *et al.* 1994; CHARLESTON *et al.* 2002; LIANG *et al.* 2008; NEILL 2013).

BVDV has been characterized as highly heterogeneous based on phenotypic and genotypic differences in the virus (RIDPATH *et al.* 1994; RIDPATH 2005). BVDV exists as two biotypes, cytopathic and noncytopathic, established by infection status in cultured cells (RIDPATH *et al.* 1994; PETERHANS *et al.* 2003; CHASE 2013). The BVDV noncytopathic biotypes are most prevalent in livestock herds, and establish chronic infections that can result in persistently infected cattle (NEILL 2013). The presence of noncytopathic strains increases the challenge of identifying the viral pathogen in fetal bovine serum, as well as infected animals.

Based on the 5' untranslated region of its genome, the virus has two genotypes, type 1 and type 2 (RIDPATH *et al.* 1994). Seventeen different BVDV type 1 isolates have been described worldwide, while 1a and 1b are the only isolates that have been reported in the U.S. (RIDPATH 2005; VILCEK *et al.* 2005; GIAMMARIOLI *et al.* 2015). Type 1b has been the predominant BVDV isolate isolated from infected cattle in the U.S., found at 45.8% of laboratory diagnostics (FULTON *et al.* 2002; FULTON *et al.* 2003; FULTON *et al.* 2005; RICHESON *et al.* 2013a). BVDV type 2 has fewer reported strains than type 1, with 2a and 2b isolates found in the U.S. (RIDPATH 2005). BVDV 2b is rarely isolated, while the other three (1a, 1b, and 2a) are found commonly in the field (RIDPATH 2005; VILCEK *et al.* 2005).

BVDV is the most financially significant viral disease of cattle worldwide (ENDSLEY *et al.* 2004; LONERAGAN *et al.* 2005; JAYNES 2015). BVDV can present as an acute

infection or as persistently infected (PI). Noncytopathic BVDV can evade the innate and adaptive immune responses if transferred to the fetus during the first trimester of pregnancy to tolerize a calf to BVDV (COLLEN AND MORRISON 2000; BRODERSEN 2014). If viral infection occurs within the first 120-day of pregnancy, it can lead to the birth of a PI calf, because the virus is able to permeate the placenta before any immune function has developed (BRUSCHKE *et al.* 1998a; PETERHANS AND SCHWEIZER 2013). PI calves will shed the virus throughout their lives and serve as viral reservoirs to infect other cattle. PI calves have often infected other cattle before being detected and removed from a herd.

BVDV infections can produce diverse clinical and subclinical symptoms depending on the virulence of the BVDV strain, the reproductive and immune status of an individual, and the age of the animal (MARTIN *et al.* 1980; LIEBLER-TENORIO *et al.* 2003b; RIDPATH *et al.* 2006; RIDPATH *et al.* 2007; RIDPATH 2015). Classic signs of BVDV infection in addition to respiratory disease include: pyrexia, diarrhea, defects of the nervous, skeletal, and immune systems, reproductive failures and gastrointestinal disease (LONERAGAN *et al.* 2005; PALOMARES *et al.* 2014b; FULTON 2015; RIDPATH 2015). However, most acute BVDV infections are subclinical (CORAPI *et al.* 1989; PALOMARES *et al.* 2014a; RIDPATH 2015), increasing the likelihood that BVDV outbreaks will go unnoticed unless combined with an environmental trigger or continual association with a PI animal. BVDV outbreaks impact the productivity and economic status of cattle populations (GLEW *et al.* 2003) and can arise from persistent or acute transient infection. Infection

caused by BVDV has been associated with host susceptibility, through immunosuppression and has been linked to secondary infections that ultimately lead to BRD (PETERHANS *et al.* 2003; PALOMARES *et al.* 2014b). The role of BVDV in BRD has not only been attributed to pathogen infection (often not observed), but also to the immunosuppression caused by BVDV, which increases susceptibility to other pathogens leading to BRD onset (CAMPBELL 2004; FULTON *et al.* 2006; RIDPATH *et al.* 2007).

BVDV has been implicated in the development of BRD both serologically and by viral isolation in cattle diagnosed with BRD (FULTON *et al.* 2002). The probability of isolating BVDV from sick calves is significantly greater than the probability of isolating BVDV from healthy calves (FULTON *et al.* 2002). In addition, BVDV is commonly isolated from cattle with lung lesions or diagnosed with BRD (FULTON *et al.* 2000). Calves diagnosed with BRD in the presence of BVDV have required longer treatment times than cattle infected with other pathogens (FULTON *et al.* 2002). BVDV associations with BRD were demonstrated through experimental infection, isolation of virus or identification of pathogen in respiratory tissues, and active infection observed through seroconversion in BRD diagnosed cattle (FULTON *et al.* 2002).

BVDV is known to affect both the innate and adaptive immune responses, but the specific effects have not been well characterized in virally-infected antigen presenting cells (LEE *et al.* 2009). Immunosuppression from BVDV infection is associated with reduced lymphocytes, increased leukopenia, decreased chemokine production, and

altered immune function (PETERHANS *et al.* 2003; LIANG *et al.* 2006; PALOMARES *et al.* 2014b) and appears to be driven by loss of an effective T-cell response in infected animals (CHARLESTON *et al.* 2002; GLEW *et al.* 2003). One hypothesis for immunosuppression post BVDV infection may be the altered protein expression in infected cells, specifically antigen presenting cells, that may affect cell processes such as apoptosis, and antigen uptake, processing, and presentation (LEE *et al.* 2009). Differences in the effects of BVDV on imune cells and cytokine production is correlated with the biotype of the infective pathogen. Certain strains of BVDV utilize mechanisms, primarily the down regulation of TLR-receptors to prevent antiviral IFN production and induce immunotolerance (PETERHANS *et al.* 2003; PETERHANS AND SCHWEIZER 2013). The viral E^{rns} protein acts as a decoy receptor, and the N^{pro} (N-terminal protease) inhibits IFN expression, as part of the mechanisms to evade the host immune response (PETERHANS AND SCHWEIZER 2013). BVDV infects numerous cell types, lymphoid and myeloid cells but specifically attacks T-cells, B-cells, and antigen-presenting cells (BRUSCHKE *et al.* 1998b; PETERHANS AND SCHWEIZER 2013), through the bovine cellular receptor CD46 (MAURER *et al.* 2004). CD46 is a complement regulator that protects host cells from complement and has been associated with a number of other diseases, including BVDV (MAURER *et al.* 2004; LISZEWSKI AND ATKINSON 2015). Certain BVDV strains suppress MHC class II expression (GLEW *et al.* 2003; CHASE 2013) and down-regulate MHC class I proteins in BVDV infected monocytes, leading to decreased antigen presentation to helper T-cells and reduced phagocytosis activity (LEE *et al.* 2009). Virulence of the BVDV infective strain can alter the Th1/Th2 response observed

post BVDV challenge (LIEBLER-TENORIO *et al.* 2002; PALOMARES *et al.* 2014a). CD4 T-cells appear to be more important than cytotoxic CD8 T-cells in the clearance of BVDV, as depletion of $CD4^+$ T-cells resulted in increased viremia (COLLEN AND MORRISON 2000; COLLEN *et al.* 2002; LIEBLER-TENORIO *et al.* 2003a).

High levels of BVDV immunity prior to entering the feedlot is frequently associated with protection against viral disease, improved productivity and greater economic benefits (FULTON *et al.* 2006); however the methodology necessary to achieve an effective immune response to BVDV is not well-defined and remain a significant issue for the feedlot industry. Both humoral and cell-mediated immune responses are capable of providing protection against respiratory infection (BAUERMANN *et al.* 2013; RIDPATH 2013), although associations between vaccine induced T-cell protection are poorly correlated with protection against the pathogen (FURMAN AND DAVIS 2015). The presence of detectable humoral and/or cell-mediated immune responses does not guarantee protection against respiratory infections. Both humoral and cell-mediated immune responses can be detected in animals vaccinated with either killed or modified live vaccine products, but the level of protection afforded by the two different immunizations varies as does the ratio of B- to T-cell activation (SANDBULTE AND ROTH 2003; STEVENS *et al.* 2009). The most effective means to mitigate BVDV infection requires implementation of a whole herd health program including: removal of persistently infected calves and maintaining BVDV protection by optimizing

immunization programs at all phases of cattle production (KELLING *et al.* 2007; FULTON 2015).

Disease Prevention

Disease prevention and treatment of feedlot cattle can be costly and in some cases vaccinations and treatments of some diseases, may be ineffective. Consumer concern about the use of antibiotics and hormones in food livestock production has become a significant component of management decisions of livestock production (BELKNAP 2015; KEYES 2015; REED 2015). Currently, vaccination is one of the most commonly used methods for disease prevention. However, variation in immune response and protection achieved by vaccinations has been observed among animals (GLASS 2004; DUFF AND GALYEAN 2007b; SALAK-JOHNSON 2007; SMITH-THOMAS 2015), and contributes to the challenges associated with studying disease and immune response in cattle.

Vaccination has been used as a prevention method to lessen the impact of BVDV infection (REBER *et al.* 2006; KELLING *et al.* 2007; FULTON 2015). In addition to immunization for prevention of BRD, antimicrobials are often used in feedlots to minimize BRD incidence (KLIMA *et al.* 2014). However, improved protection from immunization will be vital for BRD prevention to reduce the need for antimicrobials in feedlots and be in compliance with new laws and regulations. Neutralization of BVDV antigens has been well characterized, however, cross-protection between strains has been

shown to be variable and not well studied (CHASE 2013). Antigenic cross reaction has been observed between type 1 and type 2, however cross-protection within a genotype has typically been stronger than across genotypes (RIDPATH 2005). Protective immune response to BVDV pathogens has not been thoroughly characterized, to develop more complete vaccines it is crucial to understand both the immune response to the vaccination as well as the host-pathogen immune interaction.

Despite the numerous immune responses that have been measured to assess effective immunity induced by vaccination, no robust T- or B-cell assays are predictive of vaccine-induced protection (FURMAN AND DAVIS 2015). The relationship between activation of cell-mediated immunity and clinical protection produced by vaccination needs further characterization and improved assay development for the quantitation of cell-mediated responses (CORTESE *et al.* 1998).

The prevalence of BVDV observed in the field demonstrates the inadequacy of current vaccines to control the pathogen (REBER *et al.* 2006). Vaccines should contain antigens from both type 1 and type 2 BVDV strains to provide optimal protection against the variety of genotypes present in the field (LIANG *et al.* 2008). Conflicting results of the most prevalent BVDV strain have been reported, with some studies showing higher prevalence of BVDV 1b at 2:1 ratio and other studies indicating no difference in the prevalence of BVDV 1a to 1b (FULTON *et al.* 2000; FULTON *et al.* 2003; FULTON *et al.* 2006). However, based on the conflicting data and knowledge that both type 1a and 1b

strains have the potential to infect cattle, protection against both strains is needed for BRD prevention from BVDV. More than 150 vaccines have been licensed in the U.S. for BVDV prevention, the majority of commercially available vaccines are composed of various BVDV 1a and BVDV type 2 strains (FULTON AND BURGE 2000). Most USDA-licensed vaccines contain either a modified live virus or inactivated type 1a strain with different antigenic properties when compared with other type 1 and 2 strains (LIANG *et al.* 2008). The lack of BVDV 1b isolates found in vaccines creates a need for cross-protection from 1a strains is critical for prevention of disease.

Vaccination of cattle with either a killed or modified live virus (MLV) product has been a major part of BVDV prevention programs, both for prevention of persistently infected individuals and those with acute infection most often associated with BRD (FULTON *et al.* 2003). The immune response to BVDV requires both innate and adaptive immune responses (SRIKUMARAN *et al.* 2007). Antigen recognition, lymphocyte proliferation, and immune accessory cells are key to developing a protective immune response to viral pathogens (SRIKUMARAN *et al.* 2007). Activation of humoral, $CD4^+$ lymphocytes, and activation of gamma-delta T-cells were observed in calves vaccinated with the BVDV MLV vaccine even in the presence of maternal antibodies; calves receiving the killed vaccine product did not activate a T-cell response specific to BVDV (ENDSLEY *et al.* 2003). Stimulation of $CD4^+$ T-cells by vaccination is important for developing a protective immune response against a BVDV infection, and *in vivo* experiments have shown that depletion of $CD4^+$ prolong the BVDV viremia infection (COLLEN AND

MORRISON 2000; LIEBLER-TENORIO *et al.* 2003a). The immune response developed after $CD4^+$ T-cell stimulation was reported to be cross-reactive and stimulated by a larger antigen repertoire for increased epitope recognition in subsequent exposures (COLLEN AND MORRISON 2000).

The efficacy and optimization of BVDV immunization programs can be limited: by stressors that cattle endure in the transport and processing procedures, by the heterogeneity of BVDV strains to which cattle are exposed, and the timing of vaccination. Immunosuppression can result from a combination of prolonged stress and the presence of replicating BVDV from vaccines (GROOMS *et al.* 2013), this presents challenges for developing protection from vaccines (RIDPATH 2013). These challenges are due to the nature of the virus and the heterogeneity of the virus, resulting in a unique interaction between the virus and the host immune system (RIDPATH 2013). Effective BVDV vaccines should protect against viremia and minimize immunosuppression (KELLING *et al.* 2005). The optimal response to vaccination is a tradeoff to achieve the maximal immune response at the lowest physiological expense to avoid reduced performance (WAGNER 2000; RIDPATH *et al.* 2010).

BVDV vaccines are less than 100% effective, in part, because some animals do not respond to immunizations. Failure to account for animal-to-animal variation in vaccine response may be very costly due to increased disease outbreaks (GLASS *et al.* 2012). Anecdotally, *Bos indicus* cattle are less likely to become ill in the feedlot than *Bos*

taurus cattle (CUSACK *et al.* 2007), however few studies have characterized differences in the immune response between the subspecies. Environmental factors such as immune status, infection from secondary pathogens, stress and the virulence of infecting BVDV pathogen interacting with the host's genetics influence the immune response of the host to vaccination and BVDV infection (LOERCH AND FLUHARTY 1999; RIDPATH *et al.* 2013). Host variation plays a critical role in antigen recognition and lymphocyte proliferation, which are critical steps to stimulate an effective immune response (SRIKUMARAN *et al.* 2007) and antigenic differences among BVDV strains create challenges for accurate diagnostic testing with implications for vaccine strategies to prevent disease (FULTON *et al.* 2003). Improved understanding of the host immune response to pathogens and current vaccines will be critical to development of vaccines with increased efficacy and adequate protection in production environments (REBER *et al.* 2006).

Immune Response

Immune response is determined by a complex system composed of organs, specific cells, cytokines, chemokines, physical barriers, and genes networks that work together to develop the correct response to protect the host from a foreign antigen and infection (ABBAS *et al.* 2011; FURMAN AND DAVIS 2015). An effective immune response depends on both innate and adaptive immunity.

Innate Immune Response

The innate immune response is defined as the non-specific immune response and serves as the first line of defense against pathogens (PETERHANS *et al.* 2003; ABBAS *et al.* 2011; CARROLL AND BURDICK SANCHEZ 2013; HUGHES *et al.* 2014). The first line of defense is the physical barriers, these include skin, mucus and mucosal secretions, and flushing activation such as saliva and tears (ABBAS *et al.* 2011). Innate and adaptive immunity are immature in newborn calves, putting them at higher risk for disease (ENDSLEY *et al.* 2003).

The innate immune system recognizes foreign substances though pathogen-associated molecular patterns (PAMP) detected by pattern recognition receptors (PRR) (ABBAS *et al.* 2011; PETERHANS AND SCHWEIZER 2013). Toll-like receptors are one example of PRRs on the surface of immune cells such as monocytes, macrophages, and dendritic cells (ABBAS *et al.* 2011). These play an important role in the stimulation of immune response to BVDV pathogens (PETERHANS AND SCHWEIZER 2013). The PRR response initiates inflammation to activate the innate immune response and is important for early detection of infectious microbes (CHASE *et al.* 2003).

Chemokines and cytokines play an important role in stimulating the immune response to infectious pathogens. Chemokines and cytokines are proteins that are secreted by immune cells that are important for cell activation, growth, migration, and differentiation which are necessary to stimulate the immune system (FURMAN AND DAVIS 2015). Type-I

interferons are released when APCs are stimulated by various PRRs that detect viral infections (ABBAS *et al.* 2011; PETERHANS AND SCHWEIZER 2013). Viruses use evasion mechanisms to prevent the activation of the IFN system or are insensitive to the IFN response and thereby evade detection (PETERHANS AND SCHWEIZER 2013).

Adaptive Immune Response

Antigen recognition and processing by the innate immune system is a pre-requisite for activating the adaptive immune response (PETERHANS AND SCHWEIZER 2013). The adaptive immune response is composed of humoral and cell-mediated immunity and both are important for protection and clearance of viral microbes (BROCK *et al.* 2007; ROOD AND STOTT 2011; CHASE 2013). Humoral and cell-mediated immune responses to BVDV target different proteins to induce the two different types of the immune response (COLLEN AND MORRISON 2000; COLLEN *et al.* 2002). Leukopenia induced by BVDV causes a reduction in $CD8^+$ and $CD4^+$ cells, reducing the adaptive immunity cell population that is available to response to the infectious microbe (SRIKUMARAN *et al.* 2007).

Antibodies can be passed from the dam to the offspring through passive transfer in colostrum and those maternal antibodies, if present above threshold levels will protect the calf from BVDV infection (ENDSLEY *et al.* 2003). However, the presence of maternal antibodies, at given levels, can also prevent immune response to vaccination (ELLIS *et al.* 2001; ZIMMERMAN *et al.* 2006; DOWNEY *et al.* 2013). The transfer of these maternal

antibodies is critical for protection against pathogens when the immune system is immature, as immunity in young calves takes up to several weeks to months to mature and begin to develop acquired immunity (ROOD AND STOTT 2011; STEVENS *et al.* 2011)

Humoral response to vaccination has typically been used to measure the acquired immune response to vaccinations (ENDSLEY *et al.* 2003). The humoral response is linked to $CD4^+$ T-helper cells, as these cells initiate stimulation of the humoral response (COLLEN *et al.* 2002; ABBAS *et al.* 2011). MHC class II receptors present antigens to activate $CD4^+$ T-cells that stimulate B-lymphocytes to differentiate into antibody-secreting plasma cells (SRIKUMARAN *et al.* 2007). T-cells respond to cytopathic BVDV strains occurs more rapidly than to non-cytopathic strains and the strain cross-reactivity appears to be MHC-restricted (COLLEN AND MORRISON 2000; COLLEN *et al.* 2002). $CD4^+$ T cells are also important in the development of memory T cells. The memory response that is achieved by the adaptive immune response is critical for an amnestic response when challenged with pathogen exposure at a later time in life (ROOD AND STOTT 2011).

Stress

Stress has been described as a general term for the response of an individual to external influences (ROTH 1985). Response to stress is individualized and varies based on perception of the external influence, and previous experiences (SAPOLSKY *et al.* 2000). Stress can be classified as acute or chronic stress (DHABHAR 2009). Acute is stress that is

of short duration, often referred to as the fight-or-flight response (DHABHAR 2009). Acute stress typically lasts for minutes to a few hours. Chronic is long-term stress, lasting for days to weeks or longer (DHABHAR AND MCEWEN 1997). Acute stress events are suggested to prime the immune response (DHABHAR 2009; CARROLL AND BURDICK SANCHEZ 2013).

Stress has often been measured as the concentration of glucocorticoids, especially cortisol, in circulation (SAPOLSKY *et al.* 2000; CARROLL AND BURDICK SANCHEZ 2013). Acute stress can prime the immune response; where as chronic stress shifts the stress response from preparatory to suppressive (DHABHAR 2009; HUGHES *et al.* 2014). The defining point between acute and chronic stress is individually variable, and can be dependent upon the animal's perception of stress and the duration of the stress, as well as previous exposure to a stressor, the gender, genetics, and temperament (HUGHES *et al.* 2014).

Just as in humans, livestock undergo many stressful events, yet it is unclear how these stressors affect the response in cattle (HUGHES *et al.* 2014). Elevated stress hormones have been linked to decreased performance, reduced reproduction rates, and increased risk for disease (LOERCH AND FLUHARTY 1999; CARROLL AND BURDICK SANCHEZ 2013) but the literature is equivocal regarding the effects of stress on the immune response. As early as 1940, acute stress was known to enhance the immune response (SAPOLSKY *et al.* 2000) although this observation did not fit the established paradigm that stress was

immunosuppressive (SAPOLSKY *et al.* 2000; DHABHAR 2009; HUGHES *et al.* 2014). The interaction of stress and immune response is highly variable from individual to individual and influenced by the individual's perception of stress (HUGHES *et al.* 2014). Therefore, stress appears to have two modes of action on the immune response. In acute stress, the immune response is enhanced to prepare the body for an impending incident. Alternatively chronic stress suppresses the immune system and restores homeostasis to prevent a destructive hyperimmune response (SAPOLSKY *et al.* 2000).

The function of peripheral leukocytes are also negatively affected by glucocorticoid levels, which lower the number of leukocytes in circulation (SAPOLSKY *et al.* 2000). T-lymphocytes, specifically $CD4^+$ helper T-cells, are also affected, more than other lymphocytes (SAPOLSKY *et al.* 2000). However, neutrophils levels are increased with glucocorticoids (SAPOLSKY *et al.* 2000). In previous experiments, acute stress immediately before exposure to an antigen increased cell-mediated immune response, suggesting that the stress response recruits the leukocytes to the infection site and stimulates an enhanced cell-mediated response to the specific antigen presented (DHABHAR AND MCEWEN 1996). Chronic stress reverses this response and suppresses an immune response to the exposed antigen (DHABHAR AND MCEWEN 1997). Improved understanding of the interactions between the stress and immune response has the potential to improve management practices for livestock producers and to increase the performance of animals while decreasing disease risk (CARROLL AND BURDICK SANCHEZ 2013).

Justification

Genetic technologies have made large-scale, high-throughput genotyping a reality and the cattle industry has taken advantage of the new technologies to improve selection for production traits. Such advances in technology have not yet enabled improvements in disease resistance in animals, readily available alternative is improved prevention utilizing vaccination (ELLIS AND HAMMOND 2014). However, effectiveness of vaccination will require both an understanding of the genetic response of the host and more effectively characterized protective responses. The possibility of using modern tools to enhance genetic selection for improved immune response to vaccination may be more effective than selecting for disease resistant animals. Therefore the focus of this dissertation was to characterize undefined variation in BoLA genotypes, to interrogate unconventional methods of disease phenotyping following a BVDV challenge, determine the protective levels of vaccination in a BVDV challenge, and investigate subspecies differences in immune response to commercial vaccines.

CHAPTER II

CHARACTERIZATION OF THE BOVINE MAJOR HISTOCOMPATIBILITY COMPLEX

Introduction

The major histocompatibility complex (MHC) found in all jawed vertebrates is highly conserved among vertebrates (PARHAM 1999; KELLEY *et al.* 2005), and variation in the genes of the MHC is important for determining disease resistance and susceptibility (PRICE *et al.* 1999; TROWSDALE 2005). The MHC is one of the most dynamic and polymorphic regions of the vertebrate genome and is a target for continued study and research. The bovine MHC, called the bovine leucocyte antigen (BoLA) complex was discovered in the 1970's (AMORENA AND STONE 1978; SPOONER 1978).

Many of the genes in the MHC function in innate and/or adaptive immunity (PARHAM 1999; KULSKI *et al.* 2002), specifically genes are involved with antigen presentation, cytokine production, inflammation, and responses to infectious diseases (KELLEY *et al.* 2005; TROWSDALE 2005; SANTOS *et al.* 2010). The MHC contains many gene families, which evolved by gene duplication, gene conversion and recombination during vertebrate evolution (XU *et al.* 1994; AMILLS *et al.* 1998; RUSSELL 2000; GOSZCZYNSKI *et al.* 2014). Studying the MHC is performed for a number of reasons: to understand evolution of the MHC and conservation of genomic regions, to gain a better understanding of the role of olfactory receptors in mate selection, the role of natural

selection on diversity of MHC haplotypes, and improved disease resistance and vaccine efficacy (ELLIS AND HAMMOND 2014).

The MHC has been associated with susceptibility to various diseases in many species. The human MHC (HLA) is associated with more than 100 different diseases (PRICE *et al.* 1999; HALDER *et al.* 2000; HORTON *et al.* 2004; TROWSDALE 2005; ORANGE *et al.* 2011; PARHAM AND MOFFETT 2013; TROWSDALE AND KNIGHT 2013) and reproduction and mate selection (YAMAZAKI *et al.* 1976; SOMMER 2005; ZIEGLER *et al.* 2005; EIZAGUIRRE *et al.* 2009; ZIEGLER *et al.* 2010). In humans, the most important role that genotyping of the HLA has played is in transplantation and recently, avoidance of drug specific sensitivities (ILLING *et al.* 2013; PETERSDORF 2013; NORMAN *et al.* 2015). BoLA is of particular interest in animal agriculture for it's potential to improve animal health (TAKESHIMA AND AIDA 2006; ELLIS AND HAMMOND 2014). In cattle, BoLA has been associated with a number of diseases, including lymphoma, mastitis, bovine leukemia virus, somatic cell count, and tick resistance (LEWIN AND BERNOCO 1986; XU *et al.* 1993; SHARIF *et al.* 1998; PARK *et al.* 2004; UNTALAN *et al.* 2007; TAKESHIMA *et al.* 2008; YOSHIDA *et al.* 2009; MIYASAKA *et al.* 2011; CHU *et al.* 2012; THOMPSON-CRISPI *et al.* 2014). Additionally, BoLA is important for genetic selection and improvement of milk yield, growth, and reproduction (HINES *et al.* 1986; BATRA *et al.* 1989; STEAR *et al.* 1989; BEEVER *et al.* 1990; WEIGEL *et al.* 1990; MEJDELL *et al.* 1994; TAKESHIMA AND AIDA 2006).

The architecture of the MHC is composed of 3 regions designated, in order, as class II, III and I (PARHAM 1999; KELLEY *et al.* 2005; TROWSDALE 2005; TAKESHIMA AND AIDA 2006). These three regions are defined by gene content and are highly conserved among mammalian species (PARHAM 1999; KELLEY *et al.* 2005; TAKESHIMA AND AIDA 2006). BoLA maps to chromosome 23 (FRIES *et al.* 1993). Definitive genes in the class IIa region, including *DRα, DRβ, DQα,* and *DQβ,* are involved in processing and presentation of extracellular antigenic peptides to $CD4^+$ T-helper lymphocytes to initiate the humoral immune response. (TAKESHIMA AND AIDA 2006; SANTOS *et al.* 2010) Multiple *DRβ, DQα,* and *DQβ* genes have been identified with copy number polymorphisms (GROENEN *et al.* 1990; XU *et al.* 1994; GELHAUS *et al.* 1999a; GELHAUS *et al.* 1999b; RUSSELL 2000; TAKESHIMA AND AIDA 2006).

Definitive genes of Class I region of BoLA include up to 6 classical class I genes encoding highly polymorphic, ubiquitously expressed, cell-surface receptors that present intracellular peptides to $CD8^+$ T lymphocytes for interrogation of cellular health (ELLIS *et al.* 1999; TAKESHIMA AND AIDA 2006; SCHWARTZ AND HAMMOND 2015). But it is unclear if these 6 genes map to 6 different loci or map to fewer, i.e. the three known loci. The 6 classical class I genes in cattle are highly variable in the expression between haplotypes and all known haplotypes present with between 1 to 3 of the classical class I genes (PARHAM 1999; TAKESHIMA AND AIDA 2006; SCHWARTZ AND HAMMOND 2015). Haplotypes with two classical class I genes are most commonly observed in the Holstein population (CODNER *et al.* 2012).

The class III region is located between the class II and class I regions and contains a dense collection of genes with diverse functions including innate immunity and cytokine production (TAKESHIMA AND AIDA 2006). The class III region is the most conserved region of the MHC (TROWSDALE 1995), and the most gene dense region of the human genome (KULSKI *et al.* 2002; XIE *et al.* 2003). Definitive immune related genes in the class III region include: complement, tumor necrosis factors (TNF), lymphocyte antigen cluster, and heat shock protein genes (HORTON *et al.* 2004), many of which are involved in inflammatory response, innate immune response, regulation of immunity and cytokine production (KULSKI *et al.* 2002; WALLIN *et al.* 2002; KELLEY *et al.* 2005). In addition, BoLA class III region includes a cluster of the largest known gene family, highly conserved olfactory receptors (SANTOS *et al.* 2010).

Rapidly evolving pathogens have been suggested to be one of the primary drivers for the evolving MHC (KELLEY *et al.* 2005; SCHWARTZ AND HAMMOND 2015). While the MHC is highly polymorphic, the evolution of the MHC has not occurred more rapidly than other regions of the genome and perhaps evolution of the MHC proceeds more slowly than in other genes (KLEIN 1987). Trans-species evolution of MHC loci was observed in many species (MAYER *et al.* 1992) and cattle share a number of trans-species polymorphisms with buffalo (SENA *et al.* 2011). Revealing that many genes in the MHC are old, predating divergence from a common ancestor. Yet diversity within the MHC continues to be maintained through variable gene content and expression in haplotypes (BIRCH *et al.* 2006).

A better understanding of the organization and diversity of the BoLA complex is important to realize the potential of genomics for the improvement of animal health (TAKESHIMA AND AIDA 2006). In the large USDA funded Bovine Respiratory Disease Complex (BRD) Coordinated Agriculture Project, no association of BRD with BoLA was found (NEIBERGS *et al.* 2014), yet BoLA has been associated with a number of diseases and immune phenotypes in other studies (LEWIN AND BERNOCO 1986; XU *et al.* 1993; UNTALAN *et al.* 2007; YOSHIDA *et al.* 2009; MIYASAKA *et al.* 2011; CHU *et al.* 2012; LEACH *et al.* 2012; THOMPSON-CRISPI *et al.* 2014). These studies predominately used family or shallow pedigreed animal resources rather than population resources, to take advantage of strong linkage to overcome the uncertainties and breed bias of populations genotyped with SNP chips.

The bovine genome sequencing project was completed in 2009 and, like the predecessor human genome project, was predicted to revolutionize the cattle industry through the use of marker assisted selection and improved genetic predictions. But few genes with large impacts for controlling complex traits have been identified in humans or cattle (EICHLER *et al.* 2010; SAATCHI *et al.* 2014; SADEE *et al.* 2014) leading to a search for causes of "missing heritability" (ZUK *et al.* 2012). It is speculated by some that the missing heritability (i.e. the missing genetic variance) might be captured by rare variants with low minor allele frequency that are often removed from GWAS studies (MANOLIO *et al.* 2009) or in epistatic interactions of identified variants that separately account for a low proportion of variance (ZUK *et al.* 2012; MACKAY 2014). Improved understanding of

BoLA haplotypes and evolution will aid our understanding of cattle genetic diversity in general and disease resistance in particular (SCHWARTZ AND HAMMOND 2015). In this study, we used BoLA homozygotes to efficiently investigate haplotype diversity in bovine.

Material and Methods

DNA Samples

The Bovine Respiratory Disease (BRD) Complex Coordinated Agricultural Project Research Team provided DNA samples for the 770K SNP haplotype analyses (NEIBERGS *et al.* 2014; VAN EENENNAAM *et al.* 2014). DNA samples for this study were selected from 2,014 total DNA samples of Holstein calves, from California and New Mexico Holstein populations, that genotyped at 95% or greater homozygous for the 1,221 SNPs in BoLA. DNA samples were purified from 3 mL of whole blood using a Puregene DNA extraction kit (Gentra, Minneapolis, MN) and quality checked with a NanoDrop® as described (NEIBERGS *et al.* 2014). Genotypes were determined on the Illumina BovineHD Genotyping Beadchip by GeneSeek (Lincoln, NE) for 777,962 SNPs (NEIBERGS *et al.* 2014). The SNPs lie in the interval 24,642,893 - 28,679,974 on chromosome 23 based on the UMD 3.1 assembly. Names and coordinates of the 1,221 SNPs are listed in Appendix 1. Animals in the BRD research project were monitored for symptoms of BRD and assigned clinical scores based on the McGuirk scoring system (MCGUIRK 2008), using rectal temperature, cough, nasal discharge, eye discharge, and ear tilt as described by (NEIBERGS *et al.* 2014). Calves with clinical score equal to or

below 3 were classified as controls and calves with clinical scores above 3 were designated as cases (NEIBERGS *et al.* 2014). One hundred and sixty Holstein calves were identified as ≥ 95% homozygous for 1,221 SNPs in BoLA. The samples were from 62 females and 98 males, representing 90 cases and 70 controls.

BoLA haplotypes from 50K SNP chip Genotypes

We previously obtained samples for 143 homozygous BoLA haplotypes defined by 53 SNPs genotyped on the Illumina Bovine 50K Genotyping Chip and graciously contributed by Drs. Robert Schnabel and Jerry Taylor, Department of Animal Science, University of Missouri (FRITZ 2009). These 143 homozygous haplotypes were identified among animals of 26 different breeds but were over-represented for Angus and Holstein breeds. Names and coordinates of the 53 SNPs used to identify the 50K homozygous haplotypes are listed in Appendix 2. Preliminary analysis of 50K haplotypes identified 7 common Holstein haplotypes (FRITZ 2009) which were used as the framework for additional analysis of haplotypes identified from the 770K BovineHD SNP chip.

Data Analysis

SNP call frequencies were analyzed for each of the 160 "homozygous" Holstein calves using PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) (PURCELL *et al.* 2007). SNPs that were located in repeat elements were identified using the UCSC Genome Browser (http://genome.ucsc.edu) *Bos taurus* UMD 3.1 assembly (KENT *et al.* 2002). Coordinates for CNVs in BoLA identified by aCGH and/or NGS (LIU *et al.* 2008;

FADISTA *et al.* 2010; LIU *et al.* 2010; KIJAS *et al.* 2011; STOTHARD *et al.* 2011; ZHAN *et al.* 2011; BICKHART *et al.* 2012; CHOI *et al.* 2013; SHIN *et al.* 2014) were converted to the UMD 3.1 coordinates using the liftover function in the USCS Genome browser CNVs, and no call SNPs were visualized with the USCS genome data tracks (RANEY *et al.* 2014).

Haplotype analyses from genotype data for the 160 "homozygous" Holstein calves were performed using PHASE and fastPHASE (University of Washington, Seattle, WA) (STEPHENS AND DONNELLY 2003; STEPHENS AND SCHEET 2005; SCHEET AND STEPHENS 2006). Haplotypes were phased for the entire BoLA complex. Two hundred and thirty of the 1,221 SNPs in the data set were genotyped as having a no call SNP in at least one animal. All SNPs with at least one no call genotype were removed prior to haplotype analysis by PHASE. Genotypes were subsequently phased based on 991 genotype calls among 160 "homozygous" individuals. In examining the data for animals that were homozygous at 95% or greater of the 1,221 SNPs it became apparent that almost all "homozygous" animals had a small number of heterozygous genotype calls (Appendix 6). Such highly similar haplotypes were subsequently organized in haplotype groups. Haplotype frequencies were based on the haplotype groups identified in PHASE with 991 SNPs.

Phylogenetic analysis of predicted haplotypes derived from analysis of both the 770K and the shared 50K haplotype analysis was performed in MEGA 6.0.6

(http://www.megasoftware.net) (TAMURA *et al.* 2013) to evaluate the ability to identify haplotypes predicted by the 50K SNP haplotypes and predict relationships among the 770K haplotype groups. The 991 SNP haplotypes from PHASE were used to create an alignment in ClustalW and phylogenetic analysis was performed with UPGMA method with 1000 bootstrap iterations. The 50K haplotypes were then compared with the 770K haplotypes, using the 30 shared SNPs present on both platforms (Appendix 3). Haplotypes were aligned with the 30 shared SNPs in MEGA using the same method as for the 770K haplotypes.

Assessment of No Call SNPs by PCR

SNP no calls result from failed clustering of data points into homozygote and heterozygote genotypes and can arise from cryptic polymorphisms, insertions and deletions, CNVs, low quality SNP design, or locations of SNPs in repetitive DNA. No call SNPs due to indels or CNVs might obscure genetic variation important for contributing to phenotype associations. As a means to identify structural variation as a source of no call SNPs, I used PCR to test whether the sequences in the regions of no call SNPs were amplifiable and of the expected lengths. Primers were designed using Primer3Plus (UNTERGASSER *et al.* 2007) in a region approximately 500 bp up and down stream of each of 18 selected no call SNPs (Table 2.1). Primers were tested for uniqueness using UCSC In-Silico PCR (KENT *et al.* 2002) and NCBI GenBank Blastn to the UMD 3.1 reference assembly (ALTSCHUL *et al.* 1990). PCR reactions were performed in a total volume of 10 μL. The PCR reaction contained 0.5 units SIGMA

JumpStart™ *Taq* DNA Polymerase (Sigma-Aldrich, Saint Louis, MO), dNTPs at 800 μM, primers at 0.2 μM each, and SIGMA 10X PCR buffer with $MgCl_2$ at 1X concentration (Sigma-Aldrich, Saint Louis, MO). The PCR program had the following cycling parameters: initial denaturation of 95° C for 3 minutes, then 40 cycles for 95° C for 45 seconds, the annealing temperature ranged between 55 to 60°, adjusted for specific primer pairs, for 45 seconds, and 72° C for 2 minutes, followed by a 10 minute final extension at 72°C. Amplification products were visualized by electrophoresis in a 1.0% agarose gel containing 5 μg ethidium bromide.

Table 2.1. No call SNPs that were validated with PCR. The SNP name, UMD 3.1 position, the number of individuals that present with the no call, the forward and reverse primer and PCR results are listed.

SNP Name	SNP Position[a]	# IND	Forward Primer	Reverse Primer	Positive Control[b]	No Call Sample[c]	Adjacent No Call SNPs[d]	Reason for No Call[e]
BovineHD2300007040	25,335,659	7	CTGTGTTTGAGCTGTGTTGACTA	CTCTTTCACATTCCTCTGTCCTAGA	+	+	10	CNV, primers are outside the breakpoints
BovineHD2300007048 BovineHD2300007049	25,349,535 25,350,763	28 28	CTGAACTGAAGCTGTACGATATTG	ACCTGGGATCAAACTCAGAAC	+	-	10	CNV
BovineHD2300007057	25,417,035	87	CTGACACCTCCACCTCCTATACTT	ATCCCTGCTAGAAAACCATGTAGAC	-	-	10	CNV
ARS-BFGL-NGS-54047	25,426,985	2	TTCAAGTGTGAGAGGGAGAGAATA	AGTTGGTAGCCCAGGACAAGTATC	+	-	1	CNV
BovineHD4100016081	25,856,289	122	GTACACAAACCACAGAATGTA	AGTAGGATAGGGAGCCAGCAGTTA	+	+	1	CNV - DQB
BovineHD2300007650	27,652,500	19	GACTCCAGAACTTTCCAAGGACTA	GACAAGGATTGACTGAAGGGATAA	+	-/+	4	CNV - MIC
BovineHD2300007668	27,700,720	93	GGAGACACAGGAAACAAGAGTTCTA	GGCAATGGATAAGAGTTCAGGTTAT	+	-	4	CNV - MIC
BovineHD2300008005	28,448,873	121	TAGAAGGATGACAGGAGAAACTGTG	ACTGAGAGTACAAGTGAGGCATTTC	+	+	6	Primers not specific; CNV - BoLA
BovineHD2300008006 BovineHD2300008012	28,450,670 28,469,826	115 91	CCCTTTCCATCTGCCATCTA	CTTTGTCCTTGTCCCTTCAGTAA	+	+	6	CNV - BoLA
BovineHD2300008012 BovineHD2300008019	28,469,826 28,486,281	91 2	GTAGGGCAGGGAAAACAGATTAG	CACAGCAAGGGAAAACAAAATAGAC	-	-	6	Primers not specific; CNV - BoLA
BovineHD2300015634	28,494,393	2	CATCAGTTTGCCTATGTCCAAGT	GTAAGCCATGAGGGTGGAGAC	-	-	6	Poor primer design; CNV - BoLA
BovineHD2300008060	28,594,947	24	GGGAGGAGCCAGGTTATGTAG	CTGAAGCCCAAGAATGGTGATTA	+	+	1	Repeat element; Experiment error
BovineHD2300008062	28,599,086	3	CACAAACACTGCAACACAGATATTC	CCACCTCGACTATACTTTCTGATTCTA	+	+	1	Repeat element; Experiment error
BovineHD2300008064	28,602,332	1	GGGTTGCACACTTTATTAGGACATA	GCGATCTCCACAGGAAAATACTC	+	+	1	Gene family; Experimental error
BovineHD2300008070	28,609,602	20	AATAGGCAGAAGGAGGTAGCTTAGT	AGTGATGGAGCACAGAGCATTCTA	-	-	1	Gene family; Experimental error
BovineHD2300008079	28,621,623	1	GAAGCGACTCTAATTCATGGACATA	CCCGACTCTTACCACATCATCTAA	+	-	1	Gene family; Experimental error
BovineHD2300008086	28,630,914	2	GGGAGAGACCCATGTTTACAA	ACATCACTGCCTCTTCTGCTTACT	+	-	1	LINE in gene family; Exp. error
BovineHD4100016107 BovineHD2300008090 BovineHD2300008091	28,634,603 28,635,439 28,636,396	9 1 1	TTGACCAAAAAGGGCAGTAAGTAG	ACCTCCTCACACTTCTCATCTGTAG	-	-	3	Gene family; Exp. Error; poor primer design

Golden Gate Assay

In an attempt to expand the number of SNP genotypes in the haplotypes identified on the 50K SNP chip, we developed a targeted genotype assay using the custom Golden Gate platform to reanalyze the DNA samples from the Missouri homozygote samples (FRITZ 2009). The Golden Gate genotyping assay was designed in collaboration with Dr. Clare Gill (Department of Animal Science, Texas A&M University), and consisted of 445 SNPs on chromosome 23 selected from among the 1221 SNPs and the 777K SNP chip. SNP genotyping was performed at the Texas A&M AgriLife Genomics and Bioinformatics Services facility. Approximately 560 individuals were genotyped on the Golden Gate assay to identify BoLA genotypes for haplotype association analysis, and for genome-specific GWAS analyses (not this study). Among these 560 individuals were 96 samples that genotyped as BoLA homozygotes based on the 50K SNP chip (FRITZ 2009). SNPs were clustered using the Illumina GenomeStudio software v1.0 based on all 560 animals to provide relatively equal numbers of homozygotes and heterozygotes for accurate clustering and SNPs were removed based on failed accurate clustered SNP calls. Manual SNP cluster filtering removed 77 SNPs from chromosome 23. Names, coordinates, and chip index number of removed SNPs are listed in Appendix 4. The remaining 368 SNPs in BoLA were distributed across chromosome 23 from positions 26,101,031-29,483,983 (BTA 4.2 assembly).

Results

BovineHD 770K BoLA SNP Distribution

The Bovine HD 770K SNP chip contains 1,221 SNPs present but unequally distributed across BoLA (Figure 2.1). The class IIa region is approximately 2 megabases in length, spanning positions 24,642,893 - 26,861,195 on chromosome 23 and is defined by the boundary genes *TRAM2* and *BoLAII-DQβ*. Approximately 1 megabase of DNA in the class II region, extending from *BoLA-DQβ* to *Notch 4* (Class III region), is remarkably devoid of genes, containing only 8 butyrophin-like predicted genes sequences. The class II region is marked by 494 SNPs on the 770K chip. The first megabase of the class IIa region from positions 24,642,893 - 25,863,045 contains all of the functional genes mapped to BoLA class IIa and is marked by approximately 450 SNPs. The second megabase (25,863,046 - 26,861,195) with *DQβ* and the predicted butyrophilin-like sequences is marked by only 40 SNP (Figures 2.1 and 2.2). The class IIa region contains 2 regions poorly marked by SNPs, one of approximately 200 Kb at 25,268,437 - 25,425,442 and a second of approximately one megabase at positions 25,642,674 - 26,663,258. Both of these poorly marked regions are within identified CNVs.

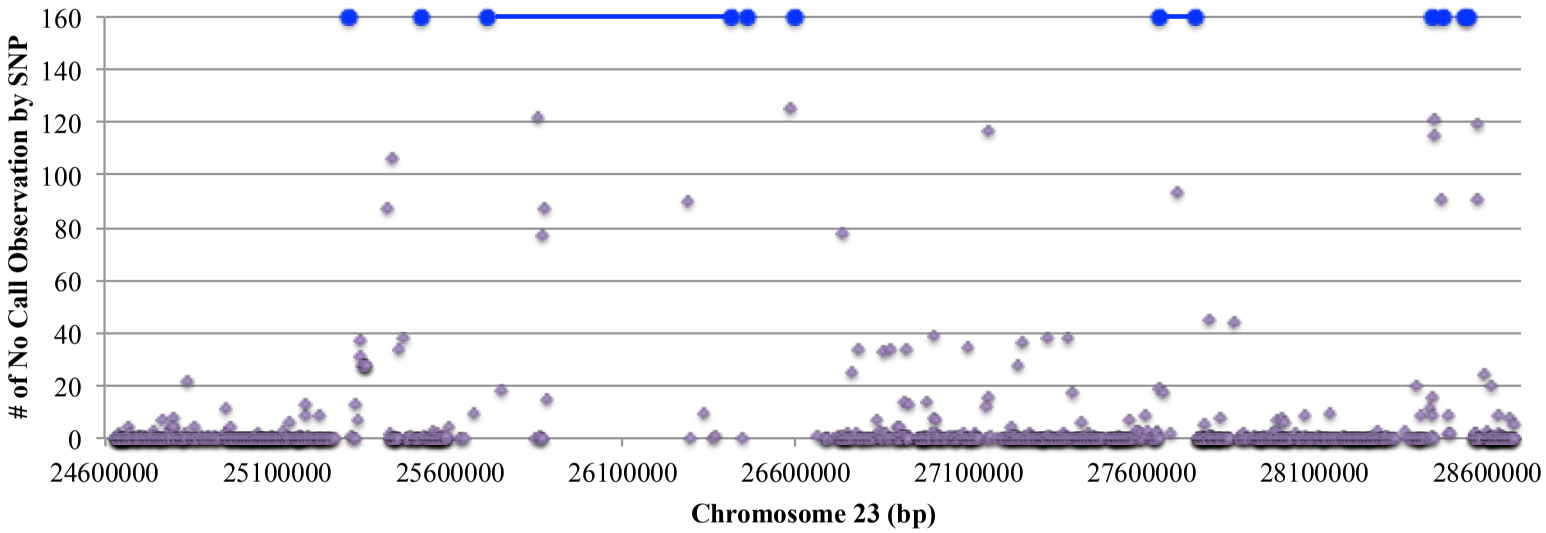


Figure 2.1. Density of MHC SNPs and Frequency of No Call SNPs in the MHC. Frequency of no calls SNPs from the Bovine HD 770K SNP chip in 160 homozygous individuals and distribution of 1,221 SNPs that span the MHC on chromosome 23, chromosome position is in UMD 3.1. The blue bars represent CNV regions defined by aCHG from the literature.

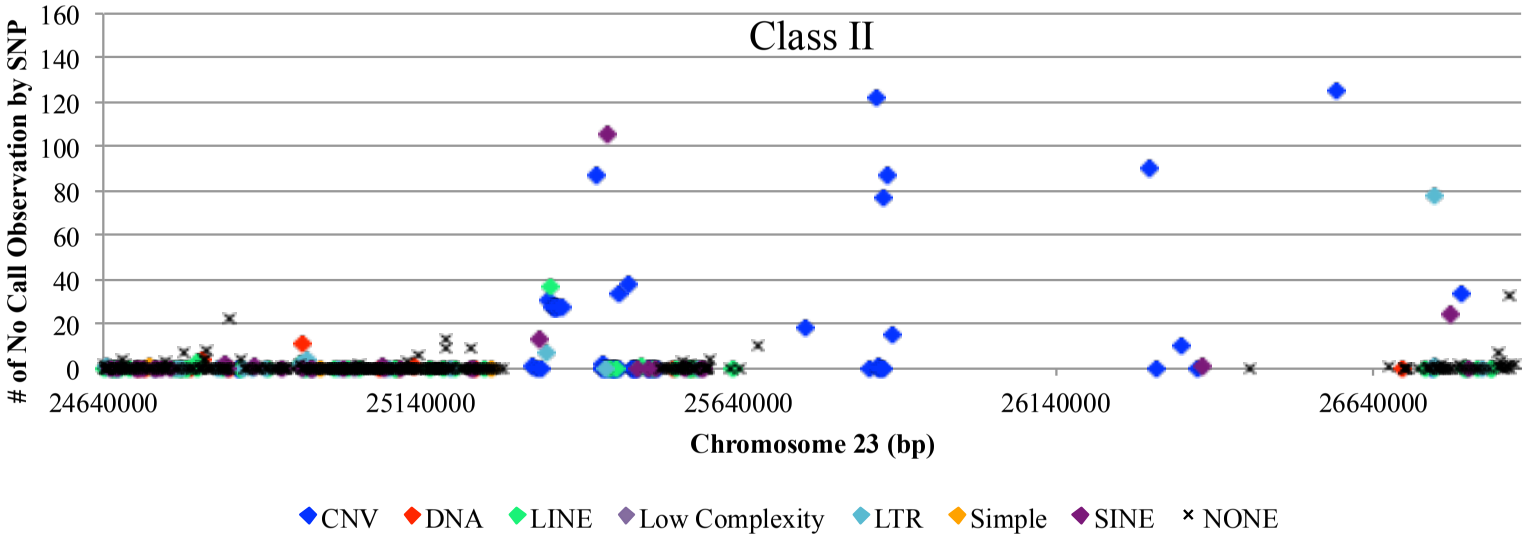


Figure 2.2. **MHC Class II No Call Frequency.** Frequency of no calls in MHC class II region of SNPs from the Bovine HD 770K SNP chip in 160 homozygous MHC individuals. The MHC class II region contained 494 SNPs, the SNPs are color coded for presence in CNVs, repeat elements (DNA, LINE, Low Complexity, LTR, Simple, and SINEs) and NONE = no repeat element or reported CNV. Coordinates for the MHC are based on UMD 3.1 build.

The class III region contains 316 SNP on the 770K chip and spans positions 26,866,572 - 27,598,351 (positions of the first and last SNP in the class III region). One small area in class III, located at 26,925,552 - 26,952,136, is unmarked by SNPs and contains no SNPs (Figures2.1 and 2.3). A second region, located at 27,133,733 - 27,184,947, is poorly marked by SNPs and SNPs present in this region have a high frequency of no calls.

The BoLA class I region is marked by 413 SNPs distributed over approximately 1 megabase at positions, 27,600,197 - 28,679,974 (the first and last SNP in the class I region) (Figures 2.1 and 2.4). The class I region has 4 poorly-marked areas. The first is 110 Kb (27,650,286 - 27,759,950) and, is associated with a CNV that is marked by only 3 SNPs (Figures 2.1 and 2.4). The second and third poorly-marked class I regions are each approximately 30 Kb long and located at positions 27,853,209 - 27,880,661 (with 1 common no call SNP (n = 44)) and at positions 28,328,861 - 28,380,703 (4 SNP, 1 rare no call (n = 3)) (Figure 2.4). A fourth poorly-marked SNP region in class I is approximately 120 Kb long (28,440,539 - 28,561,217) and contains 6 SNPs, 3 of which are predicted to be located in a CNV region with no call frequencies of approximately 50% (Figure 2.4). These 6 SNPs are located in the first half of the fourth poorly-marked region leaving another 60 Kb (28,494,394 - 28,561,217) of the class I region completely unmarked by SNPs (Figure 2.4).

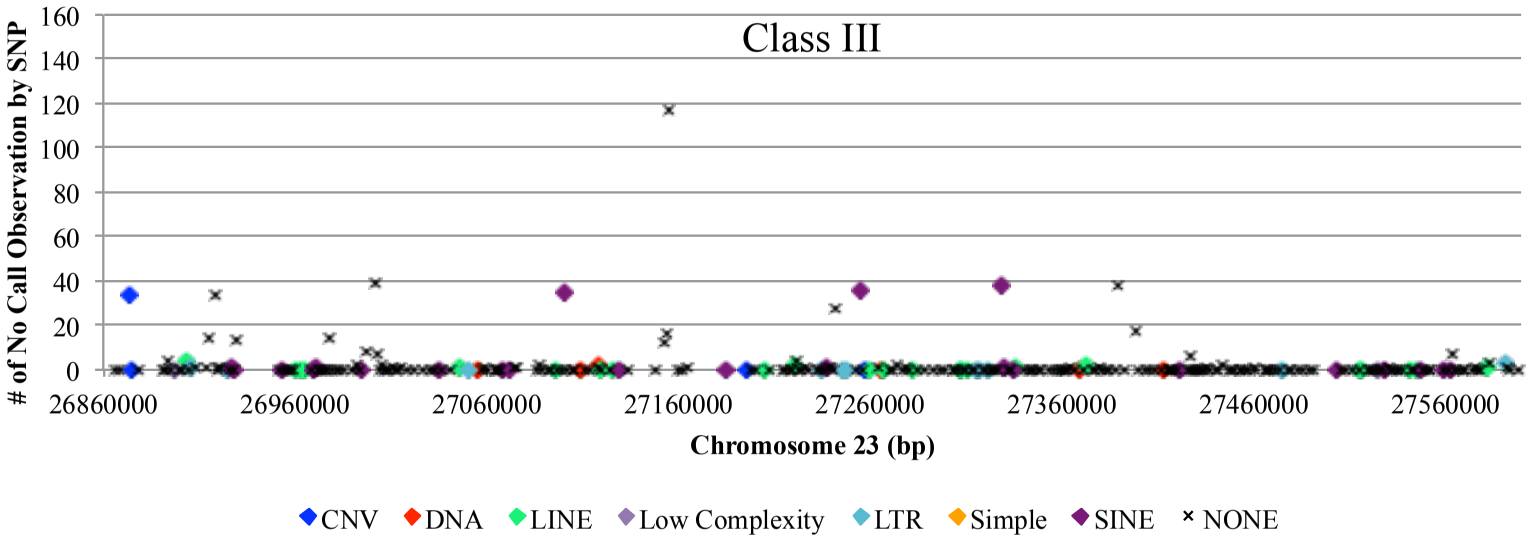


Figure 2.3. **MHC Class III No Call Frequency.** Frequency of no calls in MHC class III region of SNPs from the Bovine HD 770K SNP chip in 160 homozygous MHC individuals. The MHC class III region contained 315 SNPs, the SNPs are color coded for presence in CNVs, repeat elements (DNA, LINE, Low Complexity, LTR, Simple, and SINEs) and NONE = no repeat element or reported CNV. Coordinates for the MHC are based on UMD 3.1 build.

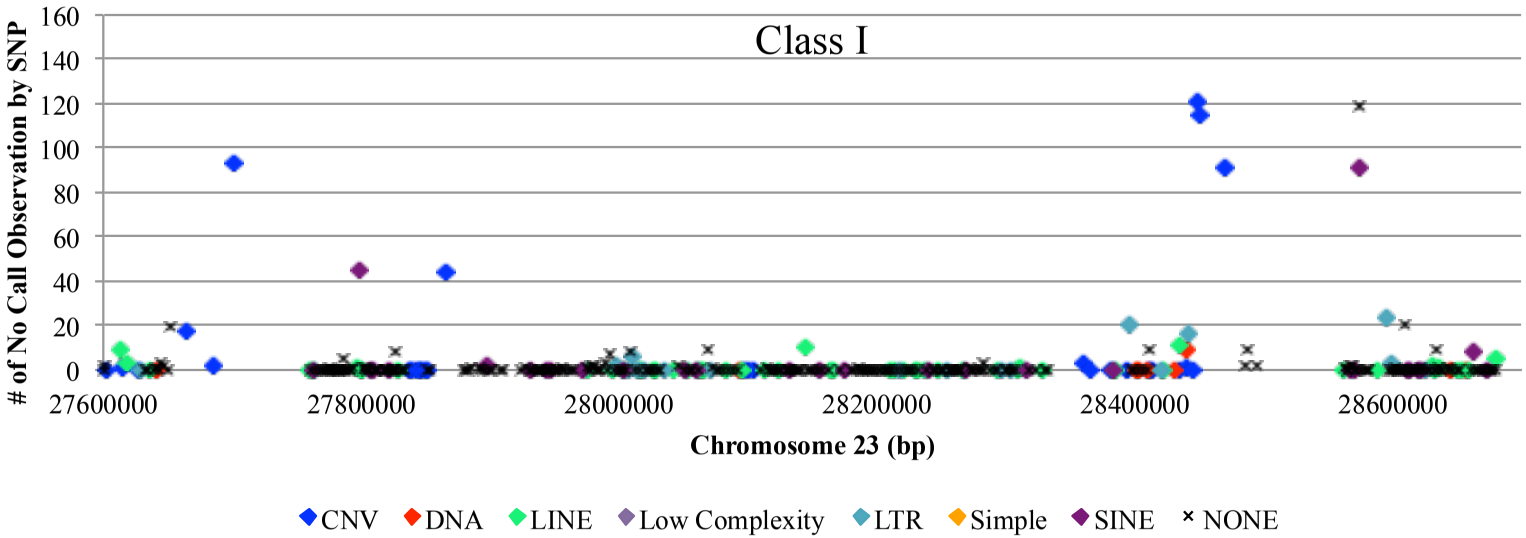


Figure 2.4. **MHC Class I No Call Frequency.** Frequency of no calls in MHC class I region of SNPs from the Bovine HD 770K SNP chip in 160 homozygous MHC individuals. The MHC class I region contained 412 SNPs, the SNPs are color coded for presence in CNVs, repeat elements (DNA, LINE, Low Complexity, LTR, Simple, and SINEs) and NONE = no repeat element or reported CNV. Coordinates for the MHC are based on UMD 3.1 build.

Frequency and distribution of No Call SNPs on the 770K SNP chip

SNP call rates were determined for each of the 1,221 SNPs across BoLA. Tabulation of no call SNPs identified a total of 230 SNPs with at least one no call genotype among the 160 animals (Figure 2.1). The remaining 991 SNPs had call rates of 100% in the 160 tested individuals. Therefore, 18.84% of all the SNPs present in BoLA had at least one no call genotype. Approximately 32% of SNPs (73 SNPs) with no call genotypes were commonly observed among the 160 animals at frequencies greater than 5% ($n \geq 8$ animals) and are designated as "common no call SNPs". The remaining 68% (n=157 SNPs) of total no call SNPs were present in less than 5% of the 160 animal cohort and are designated as "rare no call SNPs". Most (142 SNPs) of the rare no call SNPs were observed in only one of two animals and may represent rare haplotypes or be caused by experimental errors. Surprisingly, a sample from a single individual, animal 3443, contained 19 common and 54 rare no call SNPs. Among the 54 rare no calls in this sample, 39 were observed only in this animal and referred to hereafter as singlet no call SNP (Appendix 5). Of the singlet no call SNPs, 15.73% (n = 14) were adjacent to at least one other no call SNP in the same individual (Appendix 5).

The distribution of no call SNPs across BoLA might provide an indication of underlying structural variation. I determined that the proportions of no call SNPs were found at relatively equal proportions of SNPs localized to the three BoLA regions, at 19%, 20%, and 18% for classes II, III, and I, respectively (Tables 2.2 and 2.3).

Table 2.2. **Called SNPs Distributed in Repeat Elements.** Breakdown of called SNPs on the Bovine HD 770K SNP chip across the MHC by regions: class II, III, and I and the proportion of SNPs that lie in one of 7 different classes of repeat elements

	Class II	Class III	Class I
# of SNPs	494	315	412
Repeat Elements	35.63%	21.90%	33.25%
CNV	57	4	29
DNA	14	6	9
LINE	47	23	52
Low Complexity	2	2	0
LTR	20	11	18
Simple	3	0	0
SINE	33	23	29

Table 2.3. **No Call SNPs in Repeat Elements**. Breakdown of no call SNPs present in class II, III, and I and the proportion of SNPs that lie in the repeat elements and the number of SNPs that fall into the 7 different repeat element classes.

	Class II	Class III	Class I
# of No Calls	94	63	73
No Call Repeat Elements	45.74%	26.89%	41.10%
CNV	22	1	10
DNA	3	1	1
LINE	4	6	9
Low Complexity	0	0	0
LTR	6	2	6
Simple	1	0	0
SINE	7	7	4

However, the occurrence of no call SNPs was more frequent in class III and I regions at 1 SNP per 11.6 Kb and 14.8 Kb, respectively, compared to class IIa with a no call SNP at 1 per 23.5 Kb. The class IIa region contained the greatest number of common no call SNPs (n= 32) and rare no call SNPs (n= 62). BoLA class III region had nearly 3 times (n =47) as many rare no call SNPs as common no call SNPs (n = 16). Interestingly, a single no call SNP in the class III region was observed in 117 animals (73%) and the only common no call SNP observed in more than 25% of the animals in the class III region. The position of this no call SNP is near the duplicated *CYP21* loci (SKOW *et al.* 1988) and may be associated with structural variation in the duplicated region. The no call SNPs in the class IIa region clustered into the two poorly-marked regions associated with known CNVs (Figures 2.2 and 2.5). Common no call SNPs in class I also clustered into the poorly-marked regions in proximity to CNVs and SINEs (Figures 2.4 and 2.6).

The number of no call SNPs within individuals ranged from a low of 11 no call SNPs in a single individual with no singlet SNPs to a high of 73 no call SNPs in animal number 3443, 54% (n=39) of which were singlets (Table 2.4). Approximately half of the 160 animals genotyped had 16 to 18 no call SNPs on the 770K BovineHD chip; however, these 16 to 18 no calls were at different SNP locations in different individuals (Table 2.4). The number of no call SNPs was highly variable within each haplotype group suggesting that different structural polymorphism may contribute to the no call

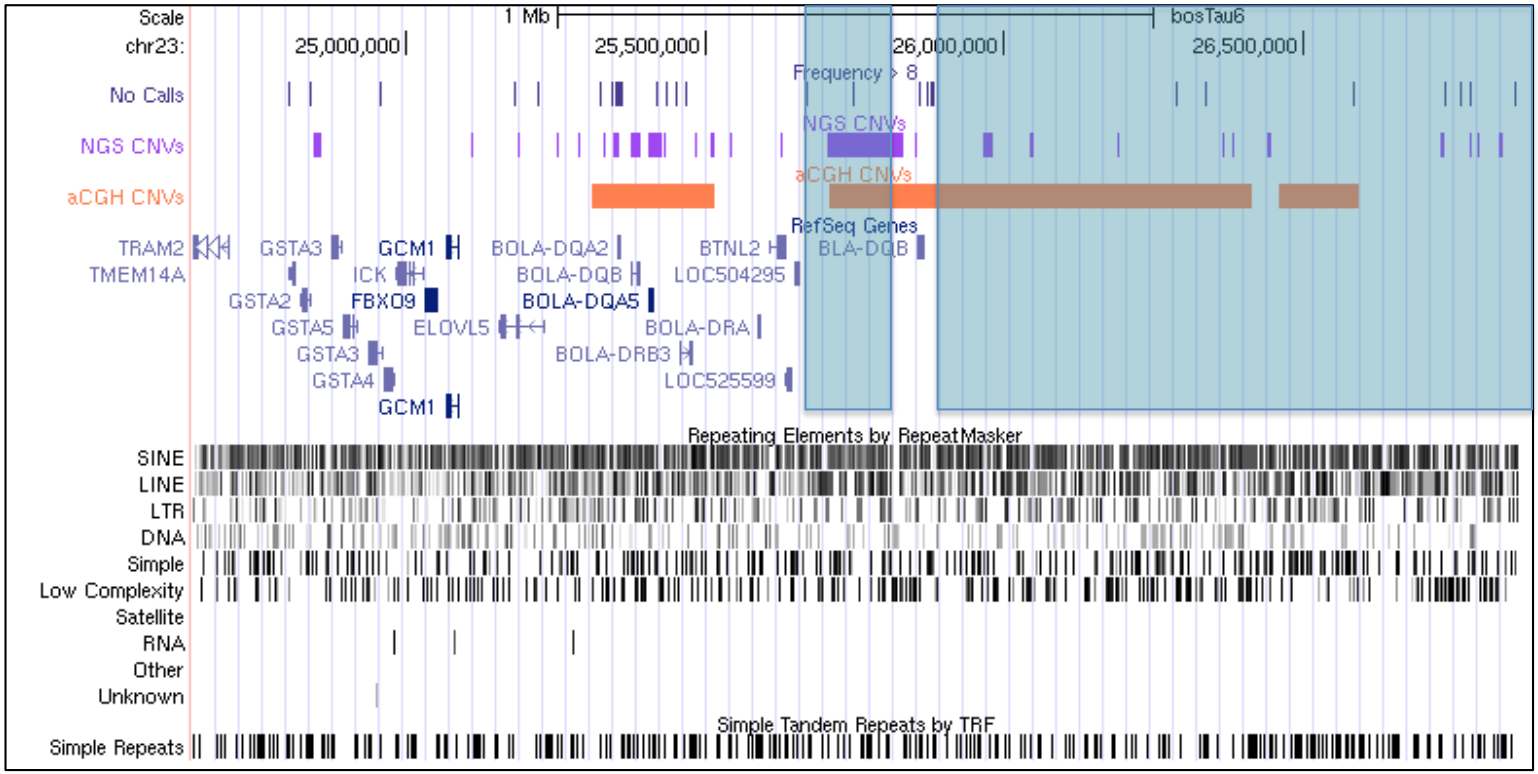


Figure 2.5. **MHC Class II No Call SNPs Genomic Relationship.** No calls SNPs in the MHC class II region overlaid onto CNVs, genes, and repeat elements in the MHC based on the UMD 3.1 assembly. Blue bars represent the no calls that were observed at a frequency > 8 (5%) in the population of 159. Purple bars represent CNVs previously described in the literature by sequencing detection. Orange bars represent CNVs previously described in the literature by aCGH detection. The annotated genes at their described location are show and various repeat elements that span the MHC. Blue boxes indicate regions poorly-marked by SNPs.

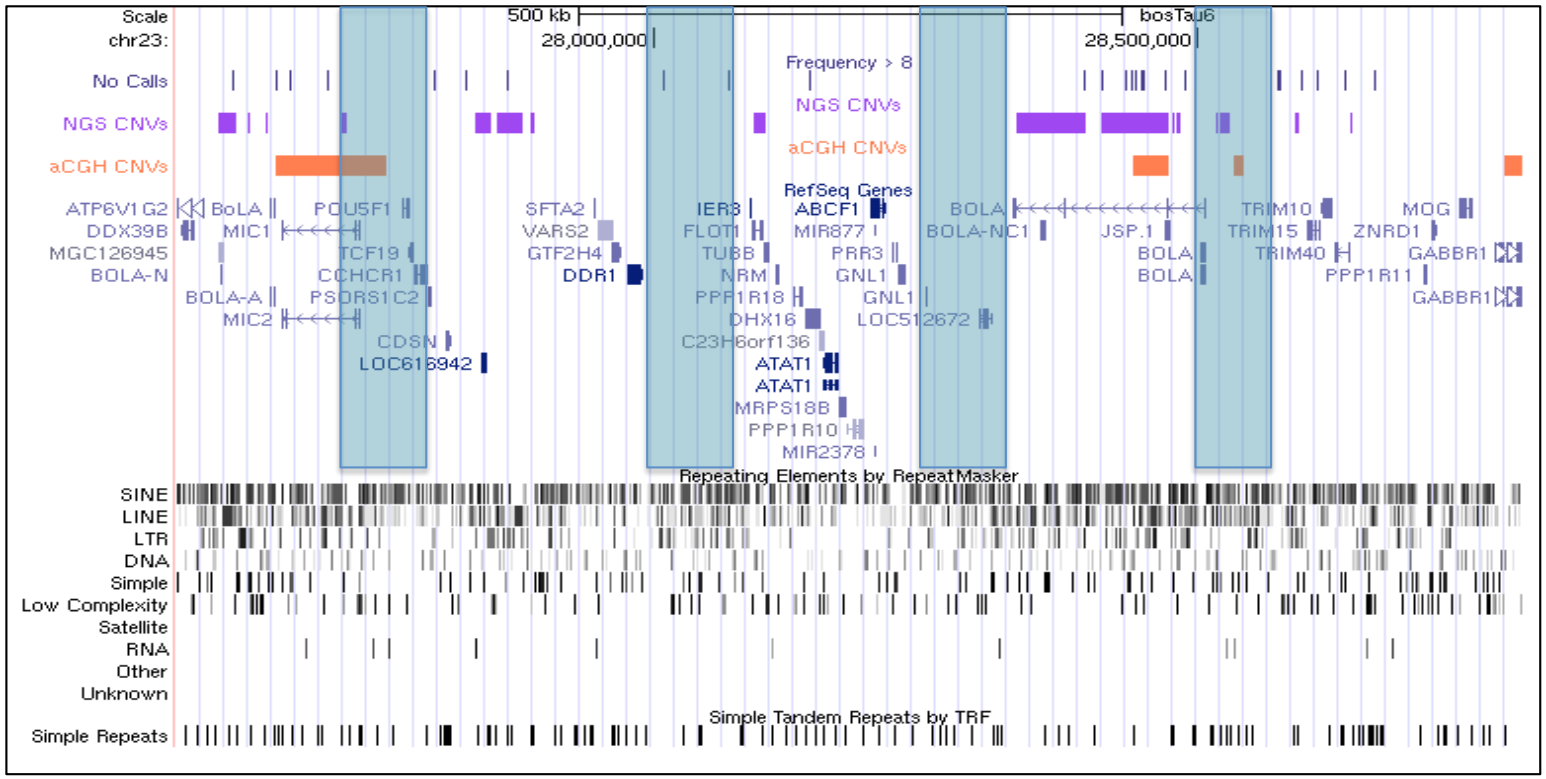


Figure 2.6. **MHC Class I No Call SNPs Genomic Relationship.** No calls SNPs in the MHC class I region overlaid onto CNVs, genes, and repeat elements in the MHC based on the UMD 3.1 assembly. Blue bars represent the no calls that were observed at a frequency > 8 (5%) in the population of 159. Purple bars represent CNVs previously described in the literature by sequencing detection. Orange bars represent CNVs previously described in the literature by aCGH detection. The annotated genes at their described location are show and various repeat elements that span the MHC. Blue boxes

Table 2.4. Frequency of No Call SNP Counts. The total number of no calls per individual were counted and the frequency of the number of no calls was calculated for the population.

No Call Count	Population Frequency
11	0.63%
13	1.25%
14	5.00%
15	8.75%
16	13.75%
17	20.00%
18	15.63%
19	7.50%
20	5.00%
21	4.38%
22	1.88%
23	2.50%
24	3.13%
25	1.88%
26	1.25%
27	2.50%
29	1.88%
30	0.63%
31	0.63%
34	0.63%
35	0.63%
73	0.63%

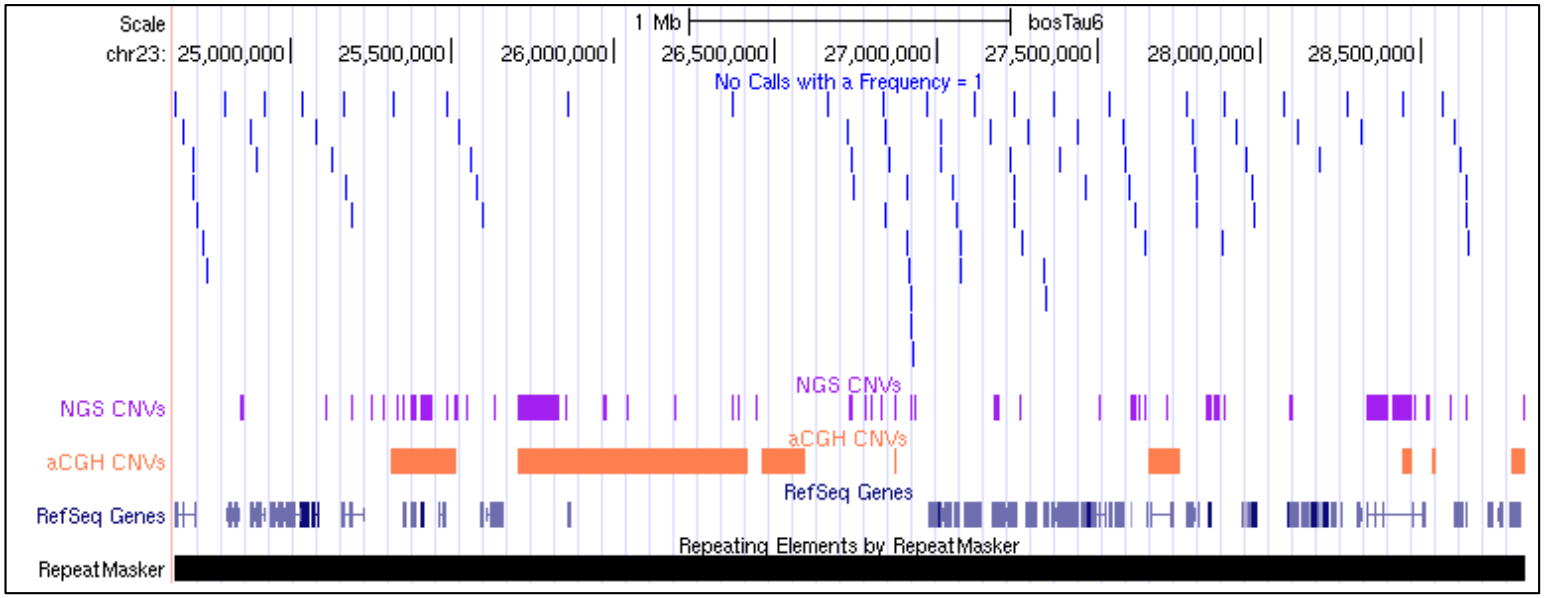


Figure 2.7. No Call SNPs in Single Animals (Singlets) Genomic Relationship. The singlet (n = 89) SNPs with no calls were plotted on the UMD 3.1 assembly with the NGS and aCGH CNVs. The reference genes and repeats have been collapsed.

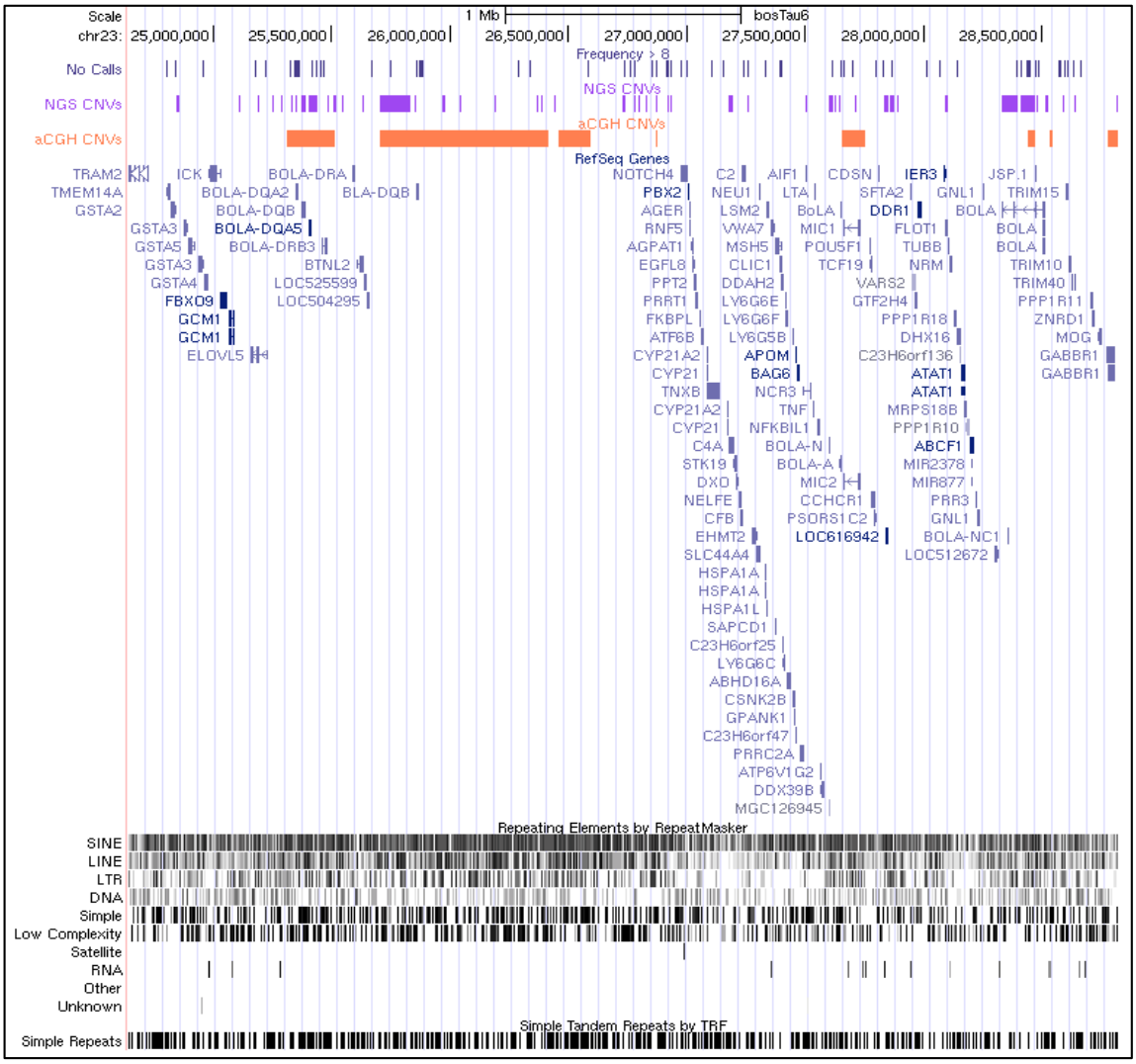


Figure 2.8. No Call SNPs UMD 3.1 Genomic Relationship. No calls SNPs overlay onto CNVs, genes, and repeat elements in the MHC based on the UMD 3.1 assembly. Blue bars represent the no calls that were observed at a frequency > 8 (5%) in the population of 159. Purple bars represent CNVs previously described in the literature by sequencing detection. Orange bars represent CNVs previously described in the literature by aCGH detection. The annotated genes at their described location are show and various repeat elements that span the MHC.

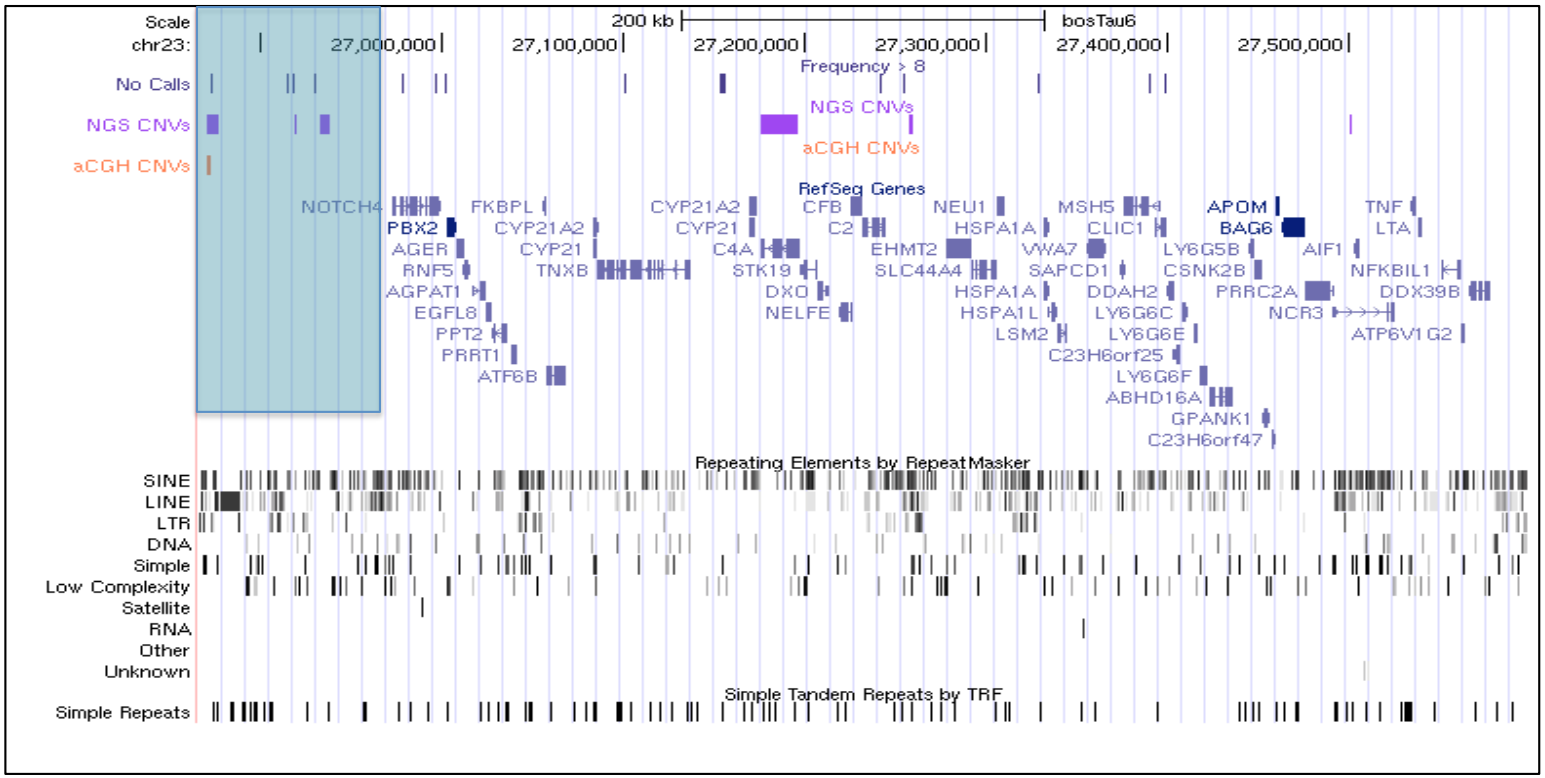


Figure 2.9. **MHC Class III No Call SNPs Genomic Relationship.** No calls SNPs in the MHC class III region overlaid onto CNVs, genes, and repeat elements in the MHC based on the UMD 3.1 assembly. Blue bars represent the no calls that were observed at a frequency > 8 (5%) in the population of 159. Purple bars represent CNVs previously described in the literature by sequencing detection. Orange bars represent CNVs previously described in the literature by aCGH detection. The annotated genes at their described location are show and various repeat elements that span the MHC. The blue box indicates that gene sparse region between class IIa and III.

genotypes. Singlet no call SNPs tended to cluster in CNV regions and were more commonly observed in the class III region than in classes IIa or I regions (Figure 2.7).

Distribution of SNPs in Repeat Elements

SNPs in all three BoLA regions associated with several different types of repeat elements (Table 2.2). Repeat elements found in BoLA are unevenly dispersed among all 3 regions (Figures 2.8, 2.5, 2.9, and 2.6). Analysis of the distribution of SNPs and repeat elements is complicated by differences in the two assemblies of the bovine reference genome. Examination the BTA 4.0 assembly reveal three distinct gaps in the distribution repeat elements and the presence of significantly more RNA repeats (RNA regulators and Piwi RNAs) than identified in the UMD 3.1 assembly (Figures 2.8 and 2.10). Near equal proportion of all SNPs in classes IIa and I were found in various repeat elements, while 12-15% fewer SNPs were found in repeat elements in class III (Table 2.2). The reduced number of repeats associated SNPS in the class III may be due to the presence of fewer repeat elements in the class III region overall when compared to the class IIa and I regions (Figure 2.8). Among the no call SNPs, more than 40% of those no call SNPs in BoLA regions IIa and I were in repeat elements, while only 26% of the no calls in the class III region were in repeat elements (Table 2.3).

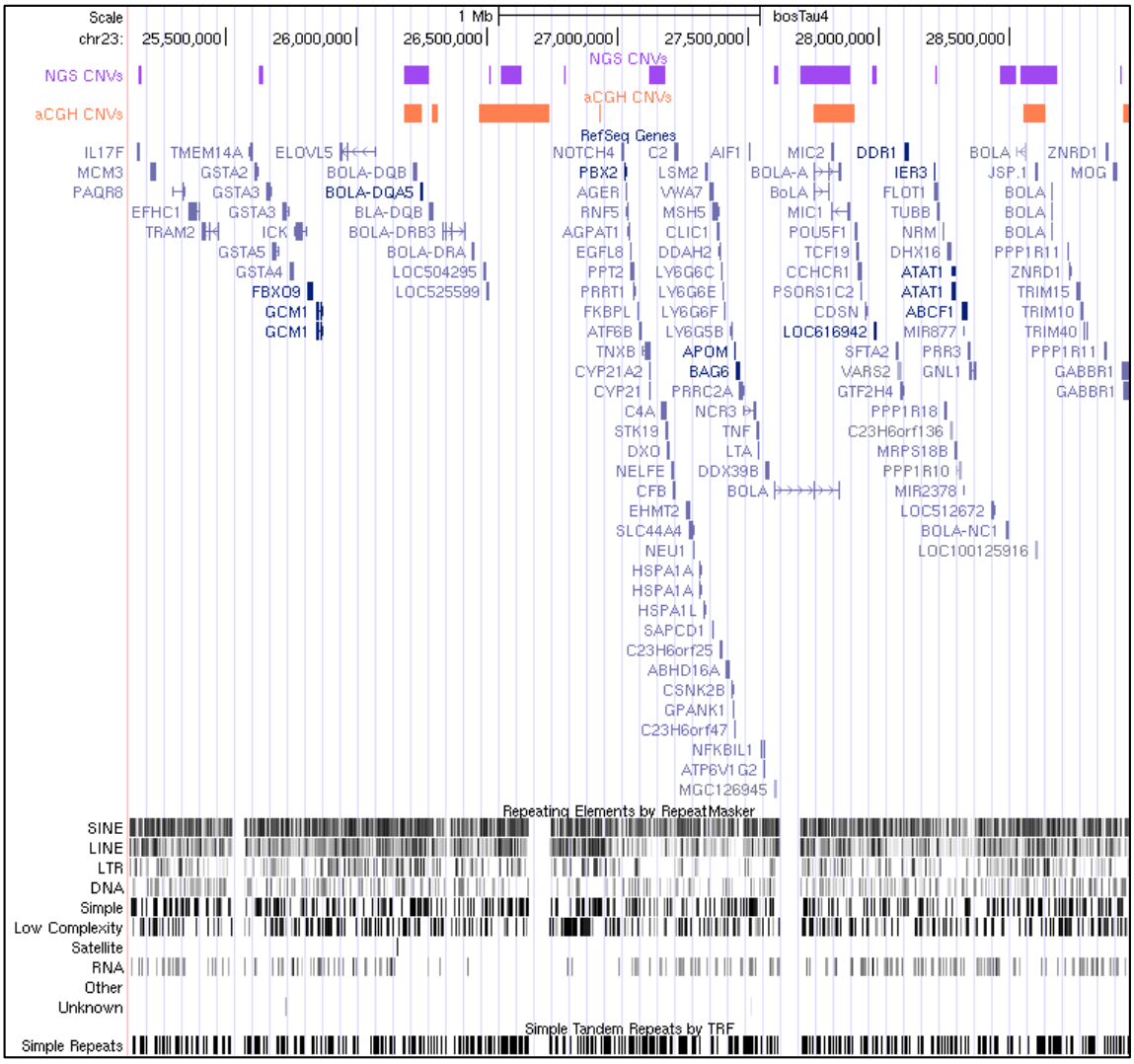


Figure 2.10. CNV Distribution on the BTA 4.0 Assembly. CNVs, genes, and repeat elements in the MHC based on the BTA 4.0 assemby. Purple bars represent CNVs previously described based on the BTA 4.0 assembly in the literature by sequencing detection. Orange bars represent CNVs previously described based on the BTA 4.0 assembly in the literature by aCGH detection. The annotated genes at their described location are show and various repeat elements that span the MHC.

In general, the largest proportions of total SNPs found in repeat elements were localized to CNVs, LINEs, and SINEs (Table 2.2). CNVs are most prominent in the class IIa region, containing 12% of the total SNPs, and approximately 50% of the SNPs within CNVs in the class IIa region contained common no call genotypes (n = 21) (Tables 2.2 and 2.3; Figure 2.2). Comparatively, 7% of the total SNPs were found in CNVs in the class I region and one third of the SNPs in CNVs were no call SNPs (Tables 2.2 and 2.3; Figure 2.5). The Class I region is enriched for LINE repeats and 40% of class I SNPs in repeat elements localized to LINEs, however only about 20% of the LINES in class I contained no call SNPs (Figure 2.4 and 2.6; Tables 2.2 and 2.3). CNVs and SINEs each accounted for 21% of the SNPs found to localize to repeat elements for class I region (Figure 2.4; Table 2.2). SNPs localized to LINEs and SINEs were observed equally in class III region, but significantly fewer SNPs present in LINEs were observed in class III compared to classes II and I (Table 2.2; Figure 2.9). No call SNPs in class III were equally distributed between LINE and SINE elements and CNVs were much less frequent in Class III (Table 2.3).

No Call SNPs and Relationship to Genes and CNVs

The locations of no call SNPs were compared to genes and previously identified CNVs in BoLA (LIU *et al.* 2008; FADISTA *et al.* 2010; LIU *et al.* 2010; KIJAS *et al.* 2011; STOTHARD *et al.* 2011; ZHAN *et al.* 2011; BICKHART *et al.* 2012; CHOI *et al.* 2013; SHIN *et al.* 2014) using both the UMD 3.1 assembly (Figure 2.8) and the BTA 4.0 assembly (Figure 2.10). CNVs in the UMD 3.1 assembly were most abundant in the class IIa

region (Figure 2.8). While the majority of CNVs called in the BTA 4.0 assembly were in the class I region. Variation in CNV size was less between the aCGH and NGS methods in the BTA 4.0 assembly (Figure 2.10). In both assemblies the class III region was relatively devoid of CNVs; this study also identified the class III region as having the fewest common no call SNPs (Figures 2.3 and 2.8). Of the no call SNPs present in the class III region, the majority were singlets (n = 31) (Figure 2.7).

Common no call SNPs also tended to cluster in or near previously identified CNVs (Figures 2.8 and 2.5). The largest identified CNV in BoLA is in the class IIa region and was identified by aCGH. This CNV encompasses the *BoLA-DQβ* gene family (Figures 2.8 and 2.5). In contrast, a CNV in the similar region in the BTA 4.0 assembly is broken into 3 parts, with the smallest of the three segments including the *BoLA-DQβ* (Figure 2.10). The largest of these three CNV segments is between the class IIa and class III genes, precisely where the poorly-marked butyrophilin gene family is located (Figures 2.1 and 2.10). Of the 40 total SNPs found in this region, 14 no call SNPs were found in the class IIa region that included the large CNV and the *BoLA-DQβ* genes (Figure 2.5) and contained the 2 most common no called SNPs (BovineHD2300007216 and BovineHD4100016081). Similarly, a CNV at the end of the first poorly marked segment in the class IIa region contains 11 no call SNPs and 4 class II genes (Figures 2.8 and 2.5). No call SNPs in CNVs identified by next generation sequencing flank the CNVs, rather than being located in the aCGH CNV region (Figure 2.5).

Similar associations of no call SNPs and CNVs were observed for the class I region. The largest number of no calls are present within a large CNV that includes a classical BoLA class I gene (Figure 2.6). Less than 10% of the no calls within the class I region are not outside a CNV region (Figure 2.6). Many of these no call SNPs that map outside of CNVs are located in regions of large gene families (Figure 2.6). Many singlet no calls were similarly found to flank CNVs in the class I region (Figure 2.7).

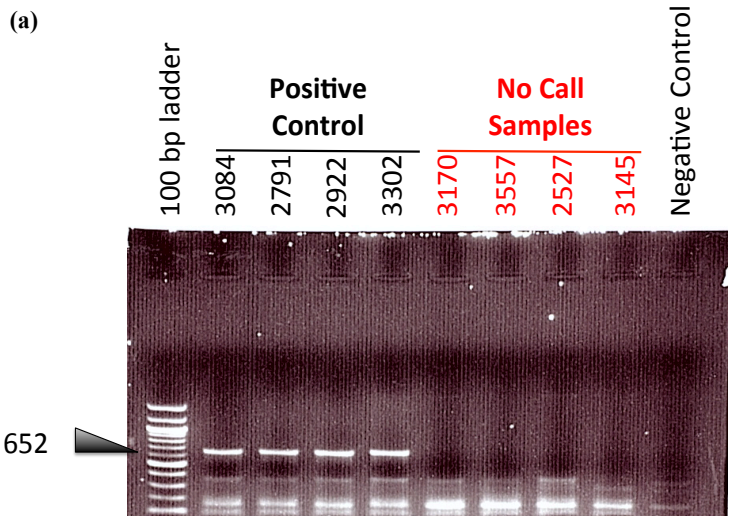
Evaluation of No Call SNPs

Eighteen no call SNPs were tested by PCR to determine whether the no call genotypes were due to deletions or genotyping errors. Tested no call SNPs and the PCR results are listed in Table 2.1. Five of the 14 SNPs tested produced no amplicon in either the no call or the positive control samples, likely due to poor primer design. Five of the 18 no call SNPs appear to be located in a deletion as no amplicon products were observed in the no call samples and the expected amplicon was observed in the positive control samples. Seven of the 18 no call samples had amplifiable product in both the no call and positive control samples. One SNP in the class I region had both amplifiable and non-amplifiable no call samples. BovineHD2300007650 was one of four adjacent no call SNPs in MIC genes of class I region, in 4 no call samples tested, 1 appears deleted while the other 3 were positive. A second no call SNP, BovineHD2300008005, produced two different size bands. In the no call animal the sample was about 1.5x the size of the positive control and no conclusion was made as to the reason for the no call genotype in this SNP. Results from the PCR testing were inconclusive and require further investigation to

confirm the reason for the no call genotype. Results of PCR analysis for two no call SNPs, BovineHD2300007668 and BovneHD2300007040, are discussed in detail.

No call SNP Bovine2300007668 (UMD 3.1 chr23: 27,700,720) was tested by PCR to determine whether the no call was due to deletion or other structural variation (Figure 2.11). This SNP is located in BoLA class I region, embedded in a region with 4 other adjacent no call SNPs (Figure 2.11b) and was observed as a no call in 93 individuals. The 4 adjacent SNPs are located in a known CNV region that includes the non-classical class I MIC genes (Figure 2.11c). The 4 individuals with no calls for this SNP represent 3 different no call SNP patterns (Figure 2.11b) perhaps indicating some level of complexity in the CNV. PCR produced amplifiable DNA in the region for the 4 control individuals and no amplification was observed in the 4 no call individuals (Figure 2.11a) consistent with an absence of this sequence in the genomes of the no call animals.

(a)



(b)

	SNP Name	BovineHD 2300007650	BovineHD 2300007654	BovineHD 2300007662	BovineHD 2300007668
	UMD 3.1 SNP Position	27652500	27665155	27685577	27700720
No Call Sample IDs	3170			X	X
	3557			X	X
	2527		X		X
	3145				X

(c)

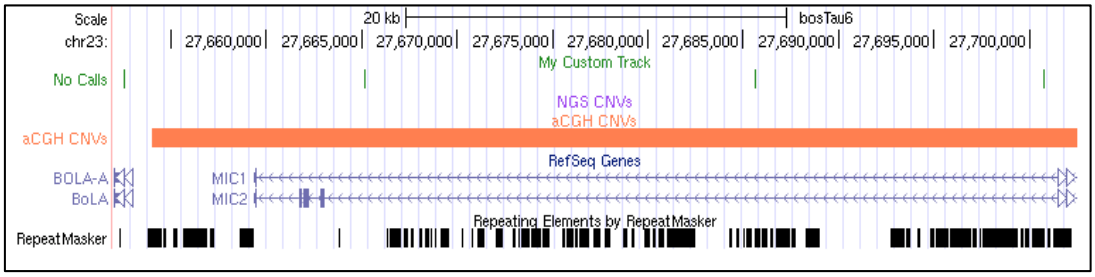


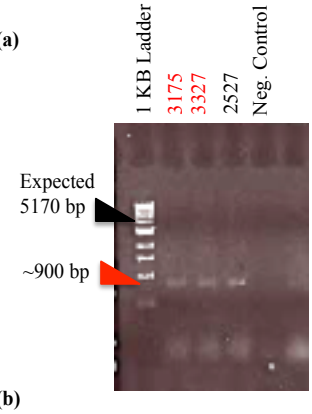
Figure 2.11. **PCR Validation of No Call SNP BovineHD2300007668. (a)** DNAs from cattle with no call genotypes at SNP marker BovineHD2300007668 (UMD3.1 chr23:27,700,720). DNAs were tested for deletions by PCR on samples from four cattle with called genotypes (lanes 2-5) and four animals with no call genotypes (lanes 6-9). A predicted PCR product of 652bp was observed in four animals with called genotypes and no PCR product in four no call animals, consistent with a deletion polymorphism at this position. **(b)** No call SNP pattern for 4 adjacent SNPs in the individuals tested for the no call. **(c)** Genomic location of adjacent SNPs relative to known CNV region and MIC genes.

A second no call SNP, BovineHD23000007040 (UMD 3.1 chr23: 25,335,659) was investigated for amplification with PCR (Figure 2.12). This SNP has a no call frequency of 4% (Appendix 5) and is one of 10 adjacent no call SNPs in the class IIa region. This SNP had the lowest frequency of any of the 10 SNPs in this region (Appendix 5). The 10 no call SNP cluster is located in a large CNV region of BoLA class IIs region (Figure 2.5) and contains class II genes *BoLA-DQα2, BoLA-DβB,* and *BoLA-DQα5*. Both of the no call animals, 3175 and 3327, had amplifiable regions (Figure 2.12).

Phenotype Association with No Call SNPs

Approximately 20% of the BoLA SNPs on the BovineHD 770K SNP chip had a no call genotype in one or more individuals and would have been eliminated from the GWAS analysis of susceptibility for BRD. It is possible that the BoLA no call SNP genotypes mark additional polymorphic regions of the bovine genome that might contribute to animal-to-animal variation in susceptibility to BRD. To test this hypothesis, I compared the distribution of BRD case and control animals among the 160 BoLA homozygotes used for this study. The proportion of cases to controls was compared for each of the 230 no call SNPs (Figure 2.13). No association between clinical phenotype and no call SNPs was observed in the case/control phenotypes. Additionally, a similar analysis was performed with the homozygous haplotype groups and demonstrated no association of BoLA haplotype with case or control was observed (data not shown).

(a)



(b)

	SNP Name BovineHD 230000:	7040	7043	7044	7048	7049	7050	7051	7052	7053	7057
	UMD 3.1 SNP Position	25332659	25341782	25343174	25349535	25350763	25351819	25350763	25354853	25361041	25417035
No Call Sample	3175	X	X	X							
	3327	X	X	X	X	X	X	X	X	X	
	2527										X

(c)

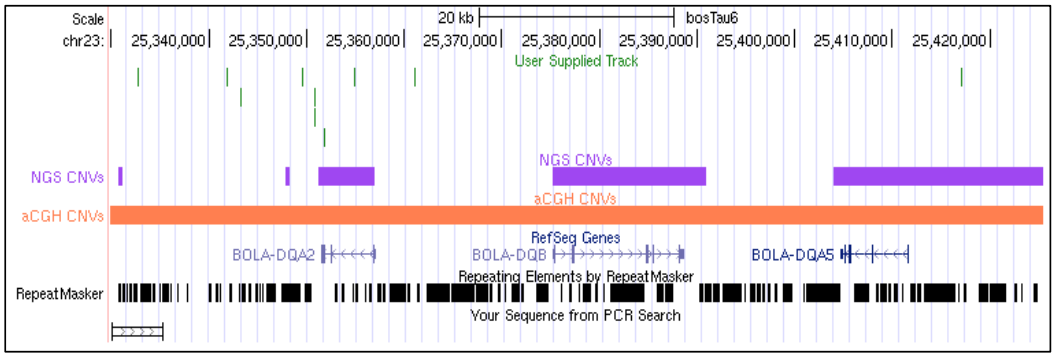


Figure 2.12. **PCR Validation of No Call SNP BovineHD2300007040. (a)** DNAs from cattle with no call genotypes at SNP marker BovineHD2300007040 (UMD3.1 chr23:25,335,659). DNAs were tested for deletions by PCR on samples from two cattle with no called genotypes (lanes 2-3) and a single animal with called genotype (lane 4). A predicted PCR product of 5170 bp was expected in the animal with the called genotype and no PCR product in two no call animals, consistent with a deletion polymorphism at this position. A PCR product of approximately 900 bp was observed in all animals. **(b)** No call SNP pattern for 10 adjacent SNPs in the individuals tested for the no call. **(c)** Genomic location of adjacent SNPs relative to known CNV region and BoLA-DQ genes. The PCR expected product is shown as the last bar under the repeats.

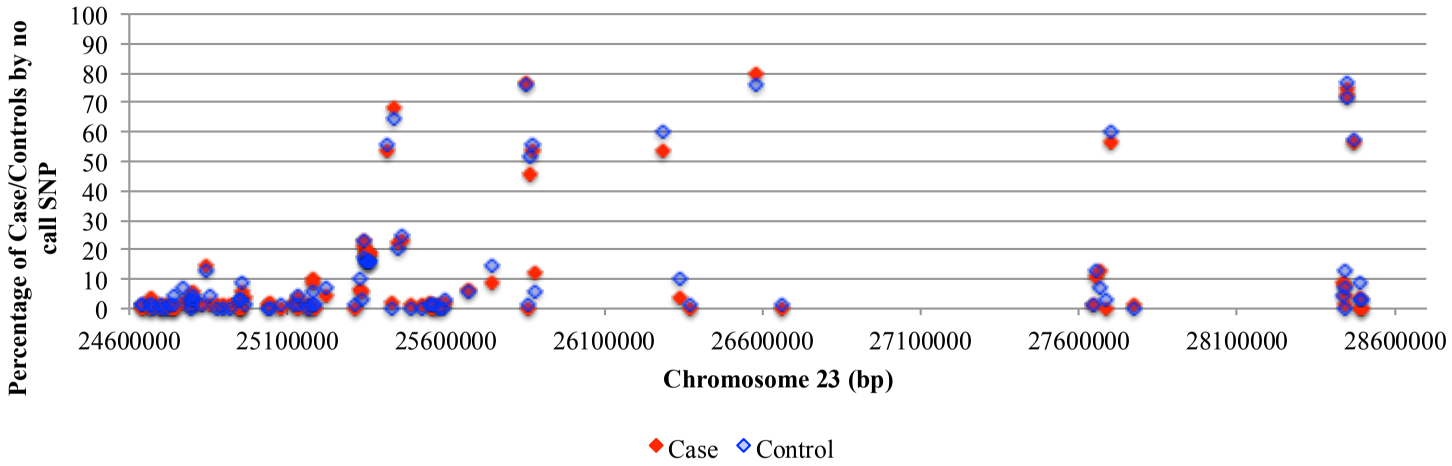


Figure 2.13. **Phenotypic Association with No Call SNPs.** The frequency of case versus control phenotypes at the 230 no call SNP positions identified in the MHC.

Frequencies of and Diversity within 770K Haplotypes

Thirty-eight different haplotype groups were identified among the 160 Holstein calves homozygous for BoLA. The frequency of individual haplotype groups in this cohort ranged from 26% for the most common haplotype, observed 83 times (320 total), to <1% (a haplotype in one animal paired with a highly similar second haplotype) for each of 18 rare haplotypes (21-38) (Table 2.5). Haplotype groups 1 and 2 were most common and together accounted for 46% of the haplotypes observed in the population. Due to the 95% homozygosity threshold, high frequency haplotypes were most often paired with a low frequency, highly similar, haplotypes or a highly similar common haplotype, i.e. 3 and 4 (Table 2.5). The six most frequent haplotype groups account for 71% of the haplotype variation observed within this Holstein population (Table 2.5).

770K Haplotypes and Relationship

Analysis by PHASE identified 38 unique haplotype groups among 160 homozygous individuals when the 230 no call SNP genotypes were removed. Among the 991 SNPs used to define the haplotype groups, 866 of those SNPs were found to be informative in this population. The two most frequent haplotype groups, 1 and 2 were 99.9% similar, differing at only a single SNP (Figure 2.14). As was seen with other highly similar haplotype groups including: 3 and 4, 5 and 6, 7 and 15, 8 and 12, 9 and 13, 10 and 16, 11 and 28, 14 and 17, 28 and 31, 18 and 19, also differed by a single SNP. The BoLA haplotypes groups segregate into three major clades within the phylogenetic tree (Figure 2.14).

Table 2.5. Haplotype Group Frequency. The frequency of a haplotype was observed among 160 Holstein individuals at 95% homozygosity for 1,221 SNPs in the MHC region of the Bovine HD 770K SNP chip. The 20 haplotype groups with a presence in 2 or more animals are listed. Haplotypes 21 to 38 were only observed 1 time in the population.

MHC Haplotype Group	Population Frequency
1	26%
2	20%
3	7%
4	7%
5	6%
6	5%
7	3%
8	3%
9	3%
10	2%
11	2%
12	2%
13	2%
14	2%
15	1%
16	1%
17	1%
18	1%
19	1%
20	1%

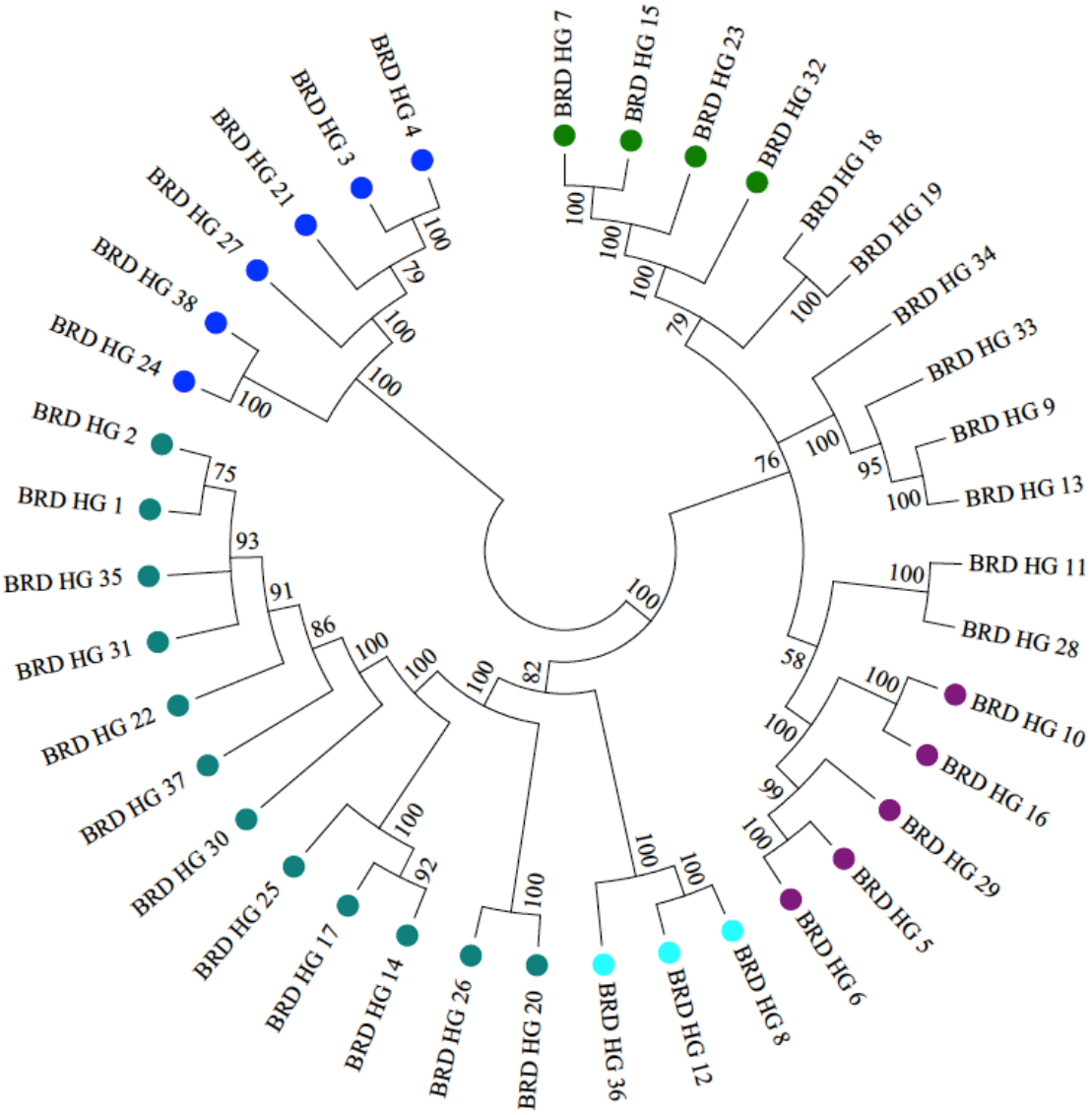


Figure 2.14. Phylogenetic Tree of BRD 770K SNP Haplotypes. Tree of 38 haplotype groups defined from 991 SNPs from the Bovine HD 770K SNP chip. Nodes are color coded based on BRD haplotypes that cluster with previously defined Holstein haplotypes from the 50K SNP chip.

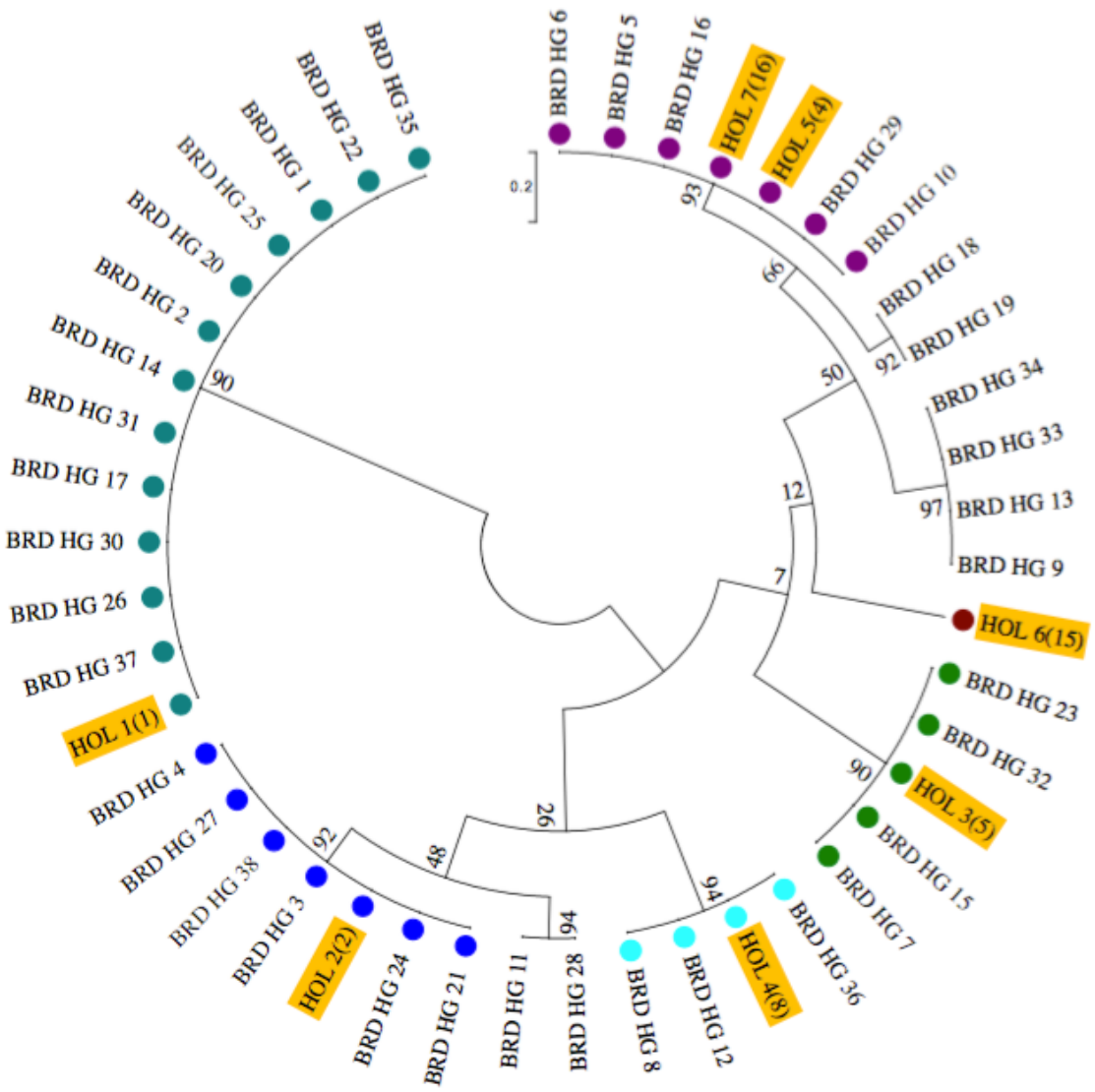


Figure 2.15. Phylogenetic Tree of Holstein Haplotypes. Haplotypes are composed of the 30 SNPs that were concordant between the 50K and 770K Illumina SNP platforms for the 7 previously defined haplotypes from the 50K SNP haplotype analysis (highlighted in gold) and the 38 haplotypes groups identified from the haplotype analysis from the BRD Holstein homozygotes.

Closely related haplotypes were often observed in the same individual, at 95% or greater homozygosity, and were treed to the same clade (Figure 2.14). The 991 SNPs used from the 770K SNP chip identify the same underlying haplotypes as were was defined by the 50K SNP identification of homozygous Holstein haplotypes (Figure 2.15).

Correlations between 50K and 770K Haplotypes

The 38 BRD Holstein 770K haplotypes were compared to the previously identified 143 haplotypes present in a large collection of BoLA homozygotes from 26 different breeds of cattle genotyped using the 50K SNP chip (Robert Schnabel and Jerry Taylor, Department of Animal Science, University of Missouri, Personal Communication) (FRITZ 2009) (Figure 2.15). Thirty SNPs are shared between the two platforms and are shown with chromosomal positions in the UMD 3.1 assembly (Figure 2.16). Fewer shared SNPs were present in the Class IIa region since this region contains few SNPs on the 50K SNP chip. The class IIa contains the greatest number of SNPs on the 770K SNP (Figure 2.16, Table 2.2). Among the shared SNPs, none were present in the large CNV that includes DQβ (Figure 2.16).

Comparison of the shared SNPs from the 7 most common Holstein haplotypes defined with the 50K SNP chip (FRITZ 2009) with the 38 BRD haplotype groups identified with the 770K SNP chip reduced the number of haplotype groups to eight (Figure 2.15). Analysis of the seven 50K Holstein haplotypes using only shared SNPs collapsed two 50K haplotypes, HOL_5 and HOL_7, into one (Figure 2.15). Six out of the seven 50K

haplotypes clustered into one of the eight 770K haplotype groups (Figure 2.15). Eight of the 38 770K haplotype groups, which composed three minor clades in the shared SNP tree analysis that did not cluster with 50K shared haplotypes (Figure 2.15). However, haplotype groups 11 and 28 collapsed to a single haplotype group and were treed to the same major clade as 50K haplotype HOL 2 (Figure 2.15). Highly similar haplotype groups 18 and 19 collapsed into a single haplotype group based on share SNP, as did highly similar haplotype groups 9, 13, 33, and 34 (Figure 2.15). These two minor clades treed to the same major clade as 50K haplotypes HOL 5 and 7 (Figure 2.15).

Validation of Illumina Golden Gate Assay

A custom Golden Gate assay was designed to independently test the predictive power of the 50K SNP chip to identify homozygous BoLA haplotypes and to determine whether a smaller SNP panel could be designed to identify the genomic variation present in BoLA. To validate the Golden Gate assay 96 individuals with 50K homozygous haplotypes were genotyped on the Golden Gate assay. Twenty-two SNPs were shared between the two platforms (Table 2.5). Of the 22 shared SNPs, only 1, ARS-BFGL-NGS-4240, accurately called the homozygous genotypes for all 96 individuals (Figure 2.17a). The other 21 markers were either unclusterable or had high heterozygous calls for individuals that previously genotyped as homozygous with the 50K platform (Figure 2.17 b-d). SNP ARS-BFGL-NGS-4203 clustered with 97% accuracy, but approximately 33% of the individuals were observed to have no call genotypes at that position; no call genotypes

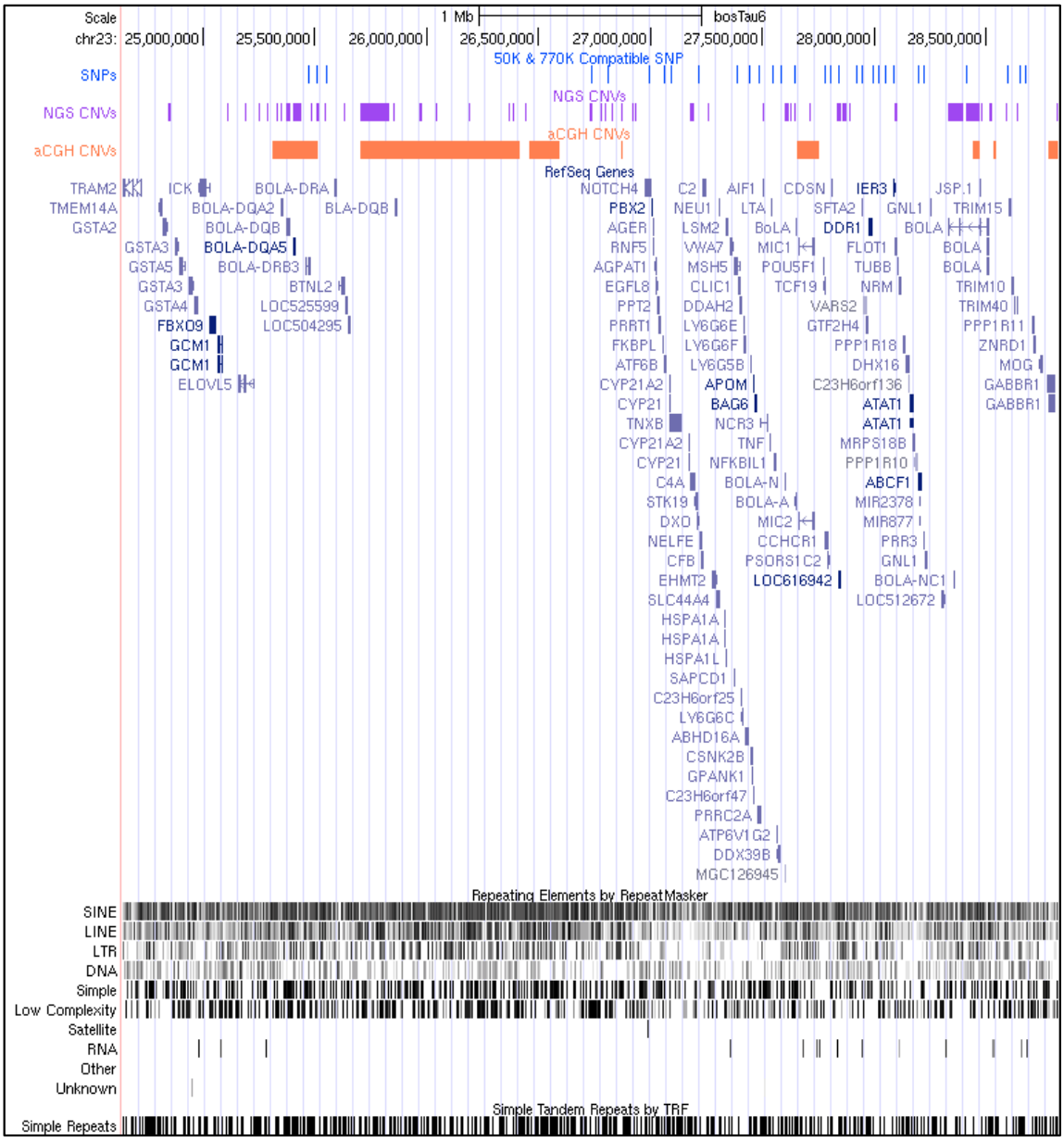


Figure 2.16. Diagram of the Compatible SNPs Between the 50K and 770K Illumina SNP Platforms. Blue bars indicate the 30 SNPs that were used to compare 50K haplotypes to 770K haplotypes. Purple bars indicate NGS CNVs from the literature. Orange bars indicate aCGH CNV regions from literature. The locations of genes are based on the UMD 3.1 assembly.

Table 2.6. Common SNPs of 50K Illumina SNP chip and Golden Gate chip. The 22 SNP that were found concordant between the 50K and Golden Gate platform used to validate the Golden Gate assay.

Common SNPs of 50K Illumina SNP Chip & Golden Gate
ARS-BFGL-NGS-4203
ARS-BFGL-NGS-109142
BTA-27247-no-rs
ARS-BFGL-BAC-28338
Hapmap47328-BTA-56087
BovineHD4100016085
ARS-BFGL-NGS-72442
Hapmap57616-rs29026690
ARS-BFGL-NGS-104658
ARS-BFGL-2116
ARS-BFGL-NGS-43021
ARS-BFGL-NGS-97747
BTA-55821-no-rs
BTA-55853-no-rs
BTA-55867-no-rs
UA-IFASA-5823
ARS-BFGL-NGS-60100
BovineHD4100016109
ARS-BFGL-NGS-96241
ARS-BFGL-NGS-95687
ARS-BFGL-BAC-35219

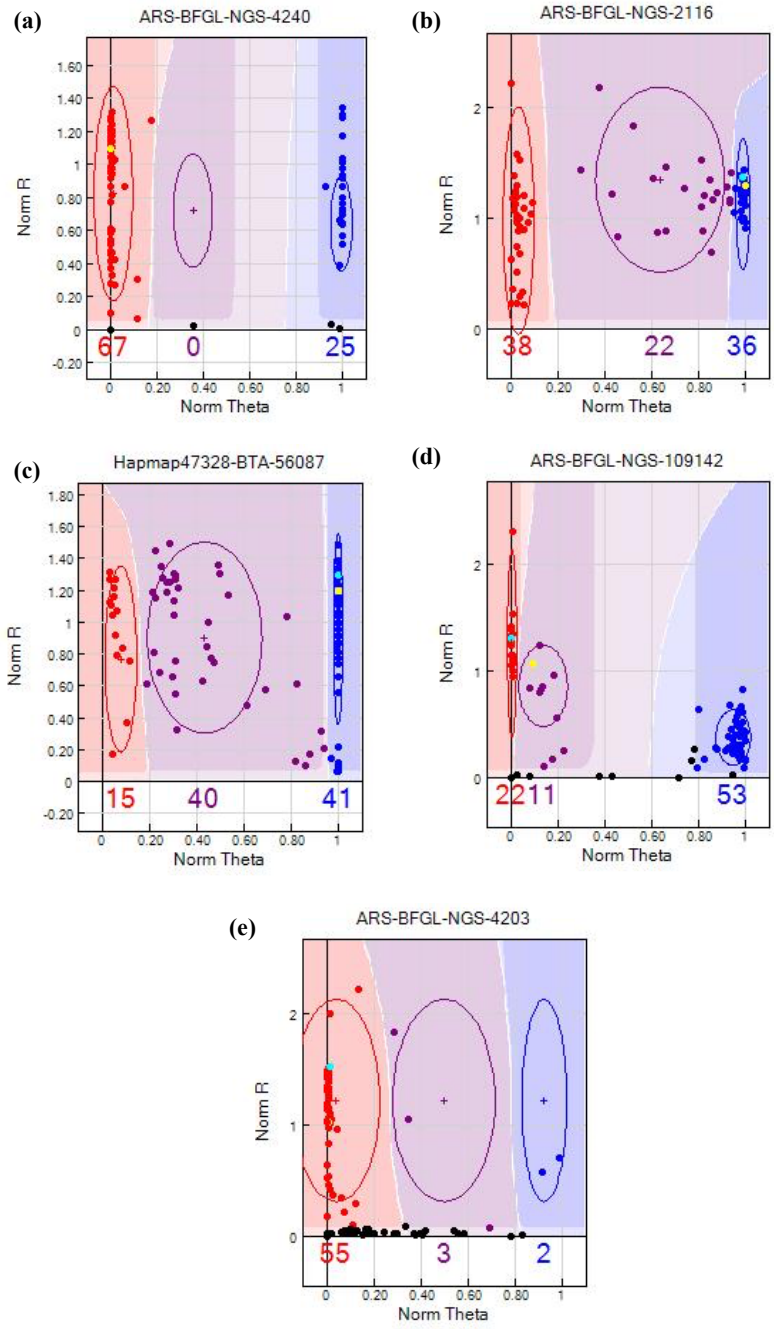


Figure 2.17. Cluster Images for Golden Gate Validated SNP. Common SNPs found on both the 50K SNP chip and the Golden Gate chip were used to validate the Golden Gate SNPs in 96 individuals previously genotyped as homozygous on the 50K SNP chip. (a) The single SNP that validated in all 96 animals. (b-d) Images of clusters for 3 SNPs that were not validated as homozygous in all 96 previously defined homozygous animals. (e) Cluster image for validate SNP with no calls for 33% of the individuals.

were observed when this SNP was genotyped on a different platform, i.e. Bovine50K chip (Figure 2.17e).

Discussion

The major histocompatibility complex is known for its structural complexities. The MHC contains many genes responsible for immunological function that likely interact with genes in other genomic regions to regulate and control diverse immune responses (TROWSDALE 1995; ZUK *et al.* 2012; AUGUSTO AND PETZL-ERLER 2015). A wide array of disease phenotypes have been observed, yet no strong single gene or variant association has been made to the MHC (ZAITLEN AND KRAFT 2012; ZUK *et al.* 2012; CAMPILLO *et al.* 2013; ELLIS AND HAMMOND 2014). Diversity of the MHC may be important for more than immune response and disease status, it may also play an important role in production and performance traits (NIKBAKHT AND ESMAILNEJAD 2015). Diversity in the MHC also appears important for many complex production traits, including reproduction, disease resistance, and immune response, olfaction and mate selection (BIRCH *et al.* 2006; EIZAGUIRRE *et al.* 2009; SANTOS *et al.* 2010; ZIEGLER *et al.* 2010; CODNER *et al.* 2012; NIKBAKHT AND ESMAILNEJAD 2015). However finding associations with the MHC may prove to be challenging in cattle due to the inadequate or spotty SNP coverage on the BovineHD SNP chip.

The lack of associations of BoLA with BRD in a large Holstein population in the Bovine Respiratory Disease (BRD) Complex CAP study (NEIBERGS *et al.* 2014), sparked our

interest characterizing in the BovineHD SNP coverage of BoLA. The SNP density in BoLA on the BovineHD chip is less than that of the average genome and has large SNP deserts which may prevent finding associations with the largest known immune related region of the genome. BoLA presents with its own challenges for designing quality probes for SNPs of interest, as BoLA has a large number of repeats (ADELSON *et al.* 2009). Nearly one third of the SNPs in BoLA are present in repeat elements and 7% of the total SNPs are associated with reported CNVs. The largest SNP desert in BoLA is located between classes IIa and III; this region is predominantly devoid of SNPs. For convenience, this region was labeled as class IIa region. It contains nearly 1 Mb of DNA with only eight predicted genes and few SNPs. A similar region in observed in HLA and ELA and the class II region in porcine is separated from classes I and III regions by the centromere (KULSKI *et al.* 2002). The SNPs that are present in BoLA in this region are in a CNV region that encompasses *BoLA-DQβ*, a gene with known copy number variation (GROENEN *et al.* 1990; XU *et al.* 1994; MARELLO *et al.* 1995; GELHAUS *et al.* 1999b; RUSSELL 2000), or other repeat elements. Illumina reports that 29,968 loci are adjacent to known polymorphisms or deletions (ILLUMINA 2015). Yet most GWAS analysis does not take this into consideration when analyzing the genotyping data. The lack of associations with BoLA in complex disease studies may be the result of more variation present in BoLA than can be captured in a traditional SNP-based GWAS. Clearly, future studies of BoLA associations warrant consideration of gene-gene interactions within BoLA or in other genomic regions and of structural variation together with SNPs to better characterize BoLA variation.

Differences in identification of CNVs between the two assemblies of the bovine genome may be due to the accuracy of the builds at the two different regions. While the two assemblies, BTA 4.0 and UMD 3.1 are mostly similar for class I, some discrepancies exist in the numbers and sizes of pseudo genes (SCHWARTZ AND HAMMOND 2015), as well as the sizes of intergenic sequences between genes and the sizes of the CNV regions. Assembly and annotation errors may be due to incorporating sequence data of more than two homologous chromosomes into the assembly (SCHWARTZ AND HAMMOND 2015). The presence of highly repetitive regions increases the challenge to correctly assemble the genome sequence.

Nearly a third of the SNPs present in BoLA were observed to have no call genotypes for 1 to 125 haplotype homozygous individuals. These SNPs had quality genotype calls in the full population and were retained in the GWAS study; however, many of the no call SNPs appear to be flanking or located in CNV regions and may be marking genomic structural variation that is otherwise not observed on the SNP chip or in GWAS. No call genotypes are excluded from typical GWAS analyses. It has been speculated that the missing heritability, especially for complex traits, may be found in the low frequency variants that are often disregarded in GWAS studies (MANOLIO *et al.* 2009). Such is also done with no call genotypes. Structural genomic variants have been associated with specific HLA alleles (NORMAN *et al.* 2015). If some of the singlet no calls mark specific genomic structural variants in BoLA then no call SNPs may be helpful in identifying structural variation present in BoLA that is important for understanding the genetic

relationship to complex traits, especially those for disease or immune response. However, no call SNPs warrants further investigation to validate the reason of the no call.

A common problem with the analysis of large genomes is the presence of repeat elements, found to compose nearly 50% of the genome (LIU *et al.* 2006; ADELSON *et al.* 2009; BURT 2009) Repeats are shown to play an important role in genome evolution, creating unstable areas that allow for the evolution of new genes (CNVs) or regulatory elements, and promote recombination events (DEININGER *et al.* 2003; LIU *et al.* 2006).

Examination of no calls by PCR suggests that a proportion of the no call SNPs are likely due to deletion polymorphisms present in CNV regions. The distribution of many no call SNPs in CNVs indicates that many of the CNVs in BoLA likely are compound and composed of combinations of several smaller CNVs. For example, the PCR analysis of no call SNPs BovineHD2300007650 and BovineHD2300007668 suggests that more than one deletion may be present in samples with no call genotypes. These SNPs maps to a known CNV region and the variation in the no call SNP patterns indicates that the CNV can exist in numerous length variants. The deletion appears to be larger than the predicted 652 bp in the four individuals tested by PCR. The four adjacent no call SNPs in this region, 27,652,500 - 27,700,720, are located in the non-classical class I MIC genes. MIC genes in other species have undergone extreme selective pressure (KULSKI *et al.* 2002) and it has been suggested that deletions and fragmentation of MIC genes may

be due to expansion of other class I genes that may be replacing the function of MIC genes (KULSKI *et al.* 2002). This would further suggest that no call SNPs in this region are more likely due to deletions and fragmentation of MIC genes due to structural variation, than to experimental error. Further investigations are required to fully determine the nature of the structural variation in this region.

DNA was successfully amplified from the two animals with no call SNP BovineHD2300007040, and the PCR products that were detected in these two animals were smaller than expected. This SNP is also associated with a large CNV region, encompassing the *BoLA-DQ* genes, which are known to have undergone gene duplications and exist as CNVs (GROENEN *et al.* 1990; XU *et al.* 1994; MARELLO *et al.* 1995; GELHAUS *et al.* 1999b; RUSSELL 2000). The smaller than predicted PCR product might suggest that the primers were poorly designed or that all individuals tested have a small, approximately 4 Kb deletion in that region or that there is an assembly error and the region of interest is smaller than indicated in the reference genome. This SNP, along with the others, warrant further investigation to determine the basis of the no call genotypes

GWAS studies using SNP-based platforms to seek associations with complex traits are questionable when the tested population is other than the population from which the chip was designed and on which the bioinformatics analysis was trained. GWAS studies have been unsuccessful in identifying the causative mutation or variants that account for large

proportions of the variance for complex traits, yet these are often the most economically important and therefore of most interest in production agriculture (ZAITLEN AND KRAFT 2012; ZUK *et al.* 2012; SAATCHI *et al.* 2014; ZHANG *et al.* 2014). As livestock industries seek to reduce the use of antibiotics, marker-assisted selection may be one way to realize improved animal health, by minimizing disease susceptibility and enhancing vaccine response (TAKESHIMA AND AIDA 2006; ELLIS AND HAMMOND 2014). A more thoroughly characterized BoLA region will be needed, as will improved technologies and analyses for gene-gene interactions (MANOLIO *et al.* 2009; ZUK *et al.* 2012; MACKAY 2014; BESSONOV *et al.* 2015).

The homozygous individuals used in this study serve as a unique resource to study the evolution, polymorphisms, structural diversity, and highly repetitive sequences that underlie haplotype diversity in BoLA. Such highly dynamic regions of the genome are refractory to contemporary sequencing methodologies unless the template can be simplified by homozygosity (NORMAN *et al.* 2015). There is evidence that haplotype structure has been conserved across mammals (VILLA-ANGULO *et al.* 2009). Conservation of trans-species polymorphisms might be due to olfactory receptor genes in MHC and their role in mate selection, to indirectly preserve diversity for immune response (SANTOS *et al.* 2010). Genotyping of homozygous or nearly homozygous individuals allows for unambiguous phasing of haplotypes when parental information is not available and the use of homozygous individuals simplifies the analysis of highly polymorphic genomic regions (NORMAN *et al.* 2015).

Comparison of homozygous haplotypes predicted from the 50K SNP chip to haplotypes identified with a 20-fold greater SNP density, revealed that the essential haplotype structure identified with the 50K chip was retained but that additional diversity was revealed within the 50K haplotypes. The haplotype diversity revealed by the 770K SNP chip still missed large amounts of uncharacterized structural variation segregating within the 38 haplotype groups. This result suggests that unidentified diversity is present within the Holstein breed and will only be catalogued by deep re-sequencing.

The haplotype phylogeny suggests that the Holstein calves in this study possibly originate from 2 major lineages, including one lineage with two significant sub-lineages within it. One sub-lineage groups has a shared HOL/ANG 50K (HOL_2/ANG_7) haplotype present. The diversity within haplotypes can give insights onto selection pressures for traits or from artificial insemination, exposure to various pathogens, and evolutionary changes for environmental effects (ELLIS AND HAMMOND 2014). Little is known about the full sequence diversity and the underlying structural variation of BoLA haplotypes (SCHWARTZ AND HAMMOND 2015) but it is evident that more variation exists among Holstein haplotypes than was described in the 50K haplotype analysis (FRITZ 2009). Therefore the haplotype structure defined for the linages present in the samples in this study may not represent all lineages of the Holstein breed.

The most frequent 770K haplotype groups (haplotypes 1 and 2) were most closely related to the most frequent haplotype, HOL 1, identified by the 50K SNP platform

(FRITZ 2009), suggesting that this is likely the most common haplotype in the Holstein breed or that the two populations from which these samples were drawn were of the same lineage. Additionally, the three 770K haplotype group clusters that were not found to match previously defined 50K haplotypes might either be one of the six low frequency haplotypes from 50K study (FRITZ 2009) or new haplotypes discovered in lineages of Holsteins not previously investigated. The frequencies of the haplotypes are population dependent, and while the HOL 1 and the BRD_HG 1 and 2 were found to be in the higher frequencies in both populations. The HOL 2 (second most frequent 50K haplotype) shared haplotype structure with the 3rd and 4th most frequent haplotypes from the BRD homozygotes. Two 50K Holstein haplotypes (2 and 7) were in the same predicted lineage, but did not tree to the same clade. Similar conserved haplotype structure has been observed in HLA haplotypes (PARHAM 1999).

The observed variability in the frequency of no call genotypes and dispersed positions of the no call SNPs within haplotype groups would suggest that structural variation is a feature of the haplotype structure and that haplotypes need to be characterized by more than SNPs. The presence of no call SNPs in CNVs and the variance in the no call genotypes fixed in single haplotype groups indicates that alternative structural variation is further defining the haplotypes within a group. It has been shown that CNVs arise more frequently that SNPs (FADISTA *et al.* 2010), and structural variation may serve to maintain and enrich diversity in BoLA, as has been suggested though variable gene content in haplotypes (BIRCH *et al.* 2006).

Identification of homozygous haplotypes can improve the genetic association testing done in disease-related studies (NORMAN *et al.* 2015) and it has been suggested that haplotype frequencies observed in cattle could be used to predict disease susceptibility (CODNER *et al.* 2012). While we did not find associations between BRD susceptibility among the haplotype groups or the no call SNPs, we did identify genotype variation that appears to correlate with genomic structural variations and repetitive DNA that are currently missed by the commercial SNP chips as currently used. The failure to detect associations in this study may be due to an insufficient population size. We also observed more variation in the haplotype groups that suggest the 1,221 SNPs that mark BoLA do not likely have enough coverage to identify all immune related variants that may be controlling genetics for complex traits.

Diversity of the MHC class I region haplotypes is largely breed specific with only a few class I haplotypes shared among breeds (ELLIS AND HAMMOND 2014) and the same condition appears to be true for haplotypes across the entire BoLA complex (FRITZ 2009). Only two of the 50K haplotypes were shared between the two most prominent breeds, Angus and Holstein. Analysis on the 770K Chip confirmed that the structure of these two haplotype groups is also shared at higher SNP densities

In conclusion, this study demonstrated that more diversity was present among "homozygous" Holstein haplotypes than was predicted from the 50K SNP chip. However, the underlying haplotype structure was consistent between the two tested

Holstein populations, suggesting that the 50K chip can predict haplotype structures within a breed but will miss some of the diversity within the breed. No call SNPs may indicate additional variation, specifically structural variation, but may also be of importance to explain some of the missing heritability for associations with complex traits. No call SNPs need further characterization to confirm that structural variation is causal or that the no calls arise from experimental error.

CHAPTER III

ANTIBODY TITERS TO VACCINATION DO NOT PREDICT LEVEL OF PROTECTION AGAINST A BVDV TYPE 1B CHALLENGE IN *BOS INDICUS* - *BOS TAURUS* STEERS

Synopsis

Subclinical illness associated with infection is thought to reduce performance and increase production costs in feedlot cattle, but underlying components remain largely unidentified. Vaccination is frequently used in feedlot settings but producers lack metrics that evaluate the effectiveness of vaccination programs. The goal of this study was to determine if serum neutralizing titers were predictive of vaccine protection in a commercial setting. Angus-Nellore steers housed in a production feedlot setting were assigned to one of three vaccine treatments: killed vaccine (KV), modified live virus (MLV) vaccine, or no vaccine, and were challenged with a noncytopathic 1b field strain of bovine viral diarrhea virus. Rectal temperature and levels of circulating lymphocytes and platelets were monitored following challenge. While no animals were diagnosed as clinically ill with respiratory disease, indicators of disease (pyrexia, lymphopenia, and thrombocytopenia) were observed. The MLV treatment elicited higher antibody titers to the vaccination than the KV, and calves in the MLV treatment had higher mean titers at challenge. The year that elicited the highest response to the vaccination and the year with the lowest frequency of phenotypic responses to the challenge were not concurrent. The MLV treatment had the highest proportion, 34.68%, of animals that were protected

against the challenge regardless of the pre-challenge antibody titer and had the fewest number of lymphopenia cases in response to the challenge. Both vaccine treatments mitigated thrombocytopenia when compared to the control treatment, and the MLV treatment reduced lymphopenia; however, these symptoms were not completely eliminated in vaccinated animals. Pyrexia was present in 40.11% of the animals, but no difference in the frequency of cases between treatments was observed. Pre-challenge vaccination response was not indicative of the level of protection nor was anamnestic antibody response correlated with healthy or sick status.

Introduction

Cattle infected with bovine viral diarrhea virus (BVDV) present with a variety of clinical and subclinical symptoms determined by the virulence of the BVDV strain and the immune status of the animal (MARTIN *et al.* 1980; LIEBLER-TENORIO *et al.* 2003b; RIDPATH *et al.* 2006; RIDPATH *et al.* 2007). BVDV has been associated with respiratory disease, reproductive failure and gastrointestinal disease (FULTON *et al.* 2006; PALOMARES *et al.* 2014b), and outbreaks reduce the productivity and economic viability of cattle populations (ENDSLEY *et al.* 2003; GLEW *et al.* 2003; PLATT *et al.* 2009). BVDV infection results in immunosuppression, predisposing cattle to secondary infections that may lead to bovine respiratory disease (BRD) (PETERHANS *et al.* 2003; SILFLOW *et al.* 2005; FULTON *et al.* 2006; RIDPATH *et al.* 2007; PALOMARES *et al.* 2014b). Some animals with acute BVDV infections have subclinical symptoms making

it challenging to identify and to determine necessary protection for disease prevention (BROCK *et al.* 2007; PALOMARES *et al.* 2014a).

High levels of BVDV immunity are associated with protection from disease, improved productivity, and economic benefits in pre-feedlot animals (FULTON *et al.* 2006). Both humoral and cell-mediated immune responses provide protection against respiratory infection (SILFLOW *et al.* 2005; BAUERMANN *et al.* 2013; RIDPATH 2013), but detectable levels of humoral and/or cell-mediated immunity do not assure protection against infections (CORTESE *et al.* 1998; FULTON *et al.* 2000; DESCÔTEAUX *et al.* 2003). While the presence of neutralizing antibodies is frequently used as a measurement of immune response to vaccination, the threshold of neutralizing antibodies required for protection against BVDV infection is unknown (BAUERMANN *et al.* 2013).

The incomplete effectiveness of BVDV vaccines is likely due to the heterogeneity among different viral strains and the unique interaction of the virus with the host immune system (GLASS *et al.* 2012; RIDPATH 2013), along with genetic and individual variation of the host (GLASS 2004; GLASS *et al.* 2012). Vaccination with either a killed or modified life virus (MLV) product is a major component of prevention programs for both persistent and acute BVDV infections (FULTON *et al.* 2003; ZIMMERMAN *et al.* 2006). It is desirable to achieve maximal response to vaccination at minimal physiological expense to avoid reduced performance (RIDPATH *et al.* 2010).

Numerous vaccine and challenge studies have been performed with differences in immune responses observed, but few have been performed in production settings to evaluate the efficacy of vaccinations in a commercial operation (LONERAGAN *et al.* 2005; HUGHES *et al.* 2014; FULTON 2015). This is important for feedlot producers to improve vaccine efficacies. This study was conducted in commercial production setting over a four-year time frame and utilized F_2 and F_3 *Bos indicus* - *Bos taurus* steers. The goals were to determine whether (1) differences existed in immune protection between killed and MLV vaccines at the same titer; (2) serum neutralizing antibody levels following vaccination were predictive of protection; and (3) current industry metrics are adequate for identification of sick animals post-pathogen exposure.

Materials and Methods

This project was reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee and the Texas A&M University Institutional Biosafety Committee. Low-stress cattle handling methods were used for this study during transportation, processing, and data collection.

Animal Population

Angus-Nellore F_2 and F_3 yearling steers were used for the study. Calves were spring-born in 2009, 2010, 2011, and 2012 and were castrated prior to weaning. Steers were weaned at approximately 7 months of age and received three clostridial vaccinations with Clostri Shield 7 (Novartis Animal Health US, Inc., Greensboro, NC) at

approximately 70 days of age, 3 weeks prior to weaning, and at weaning. Calves did not receive any vaccines against respiratory pathogens. Steers were tested for persistent BVDV infection at the Texas Veterinary Medical Diagnostics Laboratory (TVMDL; Amarillo, TX) prior to enrollment using an immunohistochemistry or antigen-capture ELISA assay on ear notch samples. All animals in this study tested negative for BVDV persistent infection and were sero-negative for antibodies against BVDV types la, 1b, and 2 prior to enrollment.

Vaccination Treatments

Steers were classified by sire and randomly assigned to one of three vaccine treatments: killed vaccine (**KV**; n = 119), **MLV** vaccine (n = 124), or control (n = 116). Animals assigned to the KV treatment received Novartis Vira-shield®, according to label directions, receiving an initial dose on day -56 or -49, and a second dose 21 days later. Steers in the MLV treatment received a single administration of Novartis Arsenal® 4.1, according to label directions, on the same day the second dose was applied to the KV treatment group. Control treatment steers received no vaccination or sham injection. The MLV vaccinated steers were housed in isolation from the other treatment groups for seven to 10 days following vaccination. Steers were assigned to one of four pens with treatment-sire groups balanced across pens.

Challenge

All steers were challenged intranasally with 5 mL of $1x10^5$ $TCID_{50}$ BVDV CA401186a (2.5 mL of inoculum per nasal passage) 25 to 35 days after booster vaccination. BVDV CA401186a was obtained from USDA-ARS National Animal Disease Center, Ames, IA (BLANCHARD *et al.* 2010).

Sample and Data Collection

Rectal temperatures were recorded on days 0, 3, 7, 10, 14, 28, and 42 post-challenge (PC).

Sera were prepared from whole blood collected on vaccination days, and days 0, 14, 28, and 42 PC and stored at –20° C until used. Serum neutralizations against cytopathic BVDV strains 1a, 1b, and 2 were performed in duplicate on serially diluted (1:4 to 1:4096) sera by TVMDL. The end titer was determined as base 2 log of the highest dilution that showed no cytopathic effect.

Whole blood was collected in EDTA vacutainers (Becton, Dickinson and Company, Franklinlakes, NJ) on days 0, 7, 14, 28, and 42 PC. Samples were shipped overnight and differential WBC counts, platelets, and red blood cell characteristics were determined on blood samples using a CELL-DYN 3700 blood analyzer (Abbott Laboratories, Abbott Park, IL) at the University of Arkansas Nutrition Laboratory (Fayetteville, AR).

Steers were observed for clinical symptoms twice daily for the first 14 days PC, and then once daily through day 42 PC. Clinical symptoms were recorded on a 6-point scale for each symptom: cough, ocular and nasal secretion, depression, diarrhea, and anorexia (STEVENS *et al.* 2011). Criteria for clinical diagnosis was a score >3 for a single symptom or a combined score ≥3 for two or more symptoms. Animals with rectal temperatures over 40.0° C, regardless of clinical scores, were treated once with tulathromycin (Zoetis, Kalamazoo, MI) according to label directions.

Calculations and Statistical Analyses

Declines in lymphocytes and platelets were calculated as the difference between the lowest circulating lymphocyte/platelet count PC and day 0 count divided by the count on day 0 PC and multiplied by 100 to generate the percent decline (RIDPATH *et al.* 2007). Anamnestic antibody response was defined as area under the curve (**AUC**) of the log base 2 titers from days 0 to 42 PC, using the trapezoidal summation method (BAXTER *et al.* 2009).

Disease phenotypes were analyzed as 2-level categorization (healthy and sick). **Pyrexia** defined as an elevated rectal temperature of 1 SD greater than day 0 PC temperature for 2 or more consecutive collection days within 14 days PC. **Lymphopenia** and **thrombocytopenia**, defined as >40% maximum decline. **Clinical** presentation was defined by presentation of pyrexia, lymphopenia, and thrombocytopenia. **Subclinical** presentation was lymphopenia and thrombocytopenia with no pyrexia.

Response to vaccination was analyzed as antibody titers in four categories (no, low, mid, and high). **No** titer included animals with no detectable titers post-vaccination (n = 153). **Low** titers were ≥2 and <4 (n = 87). **Mid** titers were ≥4 and <6 (n = 87). **High** titers were ≥6 (n = 32).

Statistical analyses were performed in SAS 9.3 (SAS Institute Inc., Cary, NC). Mixed model analysis was used for daily rectal temperature, maximum lymphocyte and platelet decline, BVDV 1b titer post-vaccination, and daily anamnestic antibody response differences with fixed effects of treatment, year and the interaction tested. Glimmix analysis was used for binary disease symptom phenotypes of: no disease symptoms, pyrexia, lymphopenia, thrombocytopenia, and clinical/subclinical presentation. Fixed effects of pre-challenge titers or BVDV 1b AUC, treatment, year, and appropriate interactions were fit into the model. Effects were considered significant at an alpha of 0.05.

Results

Response to Vaccine Treatments

Response to vaccination was measured by neutralizing antibodies on day 0, prior to the BVDV 1b challenge. The steers in the MLV treatment had higher BVDV 1b mean titers for three out of the four years compared to the KV and control treatments (Table 3.1). Highest BVDV 1b mean titers for both the KV and MLV treatments were observed in 2010 (Table 3.1). The lowest mean titer for the KV was observed in 2012, while the

lowest MLV treatment titer was observed in 2011 (Table 3.1). The peak BVDV type 2 titers were observed in 2012 for both MLV and KV treatments. BVDV 1a vaccine titers were highest in 2012 for the MLV treatment, while 2011 had the numeric peak in the KV treatment with no statistical significance between 2011 and 2012 (Table 3.1). No differences were observed in the control treatment for all BVDV genotypes as no detectable titers were measured in steers in the control treatment (Table 3.1). Year effects were observed in all BVDV antibody titers post- vaccination, however the year effects were different for the two vaccine treatments (Table 3.1). BVDV 1b antibody titers were higher for the MLV treatment than for the KV or control treatments, while the mean BVDV type 2 titers were higher in the KV treatment pre-challenge (Table 3.1).

Table 3.1. BVDV Vaccination Treatment Means Antibody Titer. BVDV 1b average neutralizing antibody titers by challenge year to the vaccination at day 0.

	N	BVDV 1b Titer (SD)	BVDV 1a Titer (SD)	BVDV 2 Titer (SD)
KV				
2010	28	3.29 (1.67)[a]	0.96 (1.53)[a]	2.14 (1.74)[a]
2011	33	2.73 (2.10)[a,c]	2.85 (2.24)[b,c]	3.27 (2.13)[b]
2012	30	1.63 (1.35)[b]	2.43 (1.57)[b,d]	3.57 (2.16)[b]
2013	28	2.29 (1.88)[b,c]	1.14 (1.63)[a]	2.18 (2.04)[a]
MLV				
2010	25	5.64 (1.38)[d]	2.32 (1.77)[b,e]	1.20 (1.26)[c]
2011	34	3.00 (1.48)[a]	2.03 (1.34)[d,e]	0.91 (1.26)[c]
2012	33	4.45 (1.23)[e]	3.33 (1.22)[c]	2.52 (1.42)[a]
2013	32	4.16 (1.53)[e]	1.34 (1.62)[a]	1.09 (1.44)[c]
Control				
2010	23	0.00 (0.00)[f]	0.00 (0.00)[f]	0.00 (0.00)[d]
2011	33	0.00 (0.00)[f]	0.00 (0.00)[f]	0.06 (0.35)[d]
2012	32	0.00 (0.00)[f]	0.09 (0.53)[f]	0.00 (0.00)[d]
2013	28	0.00 (0.00)[f]	0.07 (0.38)[f]	0.00 (0.00)[d]

Superscripts indicate difference across years within a treatment and across treatments at $P < 0.05$ within a column (BVDV genotype).

The distributions of antibody titers between the two vaccine treatments were different (Figure 3.1; Figure 3.2). Responses to the vaccines showed a bell-shaped curve in response to the KV for all years, while in 2010 more animals in the MLV treatment exhibited high titers than no or low titers (Figure 3.1). The KV treatment elicited more no and low titer responses than mid and high responses (Figure 3.1a). Alternatively, the MLV treatment elicited mid and high titers with fewer no titer responses (Figure 3.1b). The distributions of raw titer values for the two vaccine treatments are presented in Figure 3.2. The titers produced by the MLV treatment were evenly distributed with a titer of 3 at the peak of 2011 and 2013 challenge years and a peak titer of 4 in 2010 and 2012 (Figure 3.2b). The 2010 response to the KV was a bell curve similar to the response observed for the MLV treatment, however higher numbers of no responders were observed in 2011 to 2013 with the greatest proportion of the animals in those years in the no and low response groups (Figure 3.2a).

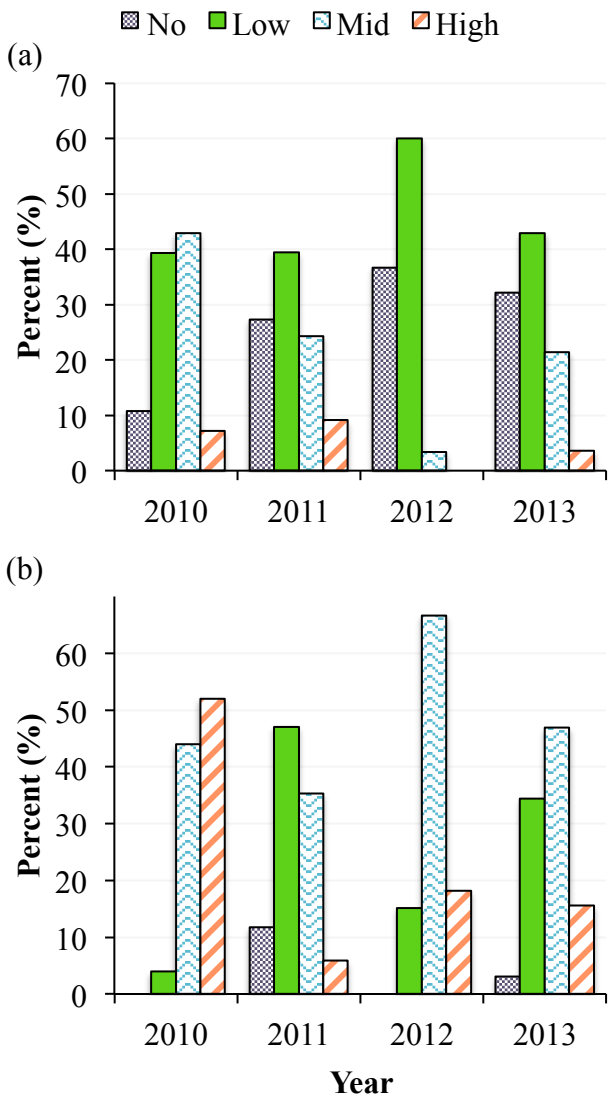


Figure 3.1. **Antibody Titers by Year.** Distribution of BVDV 1b antibody titers in steers at day 0 (No: titer = 0; Low: 2 ≤ Titer < 4; Mid: 4 ≤ Titer < 6; and High: Titer ≥ 6) to (a) Killed Vaccine (KV) and (b) MLV vaccine by year.

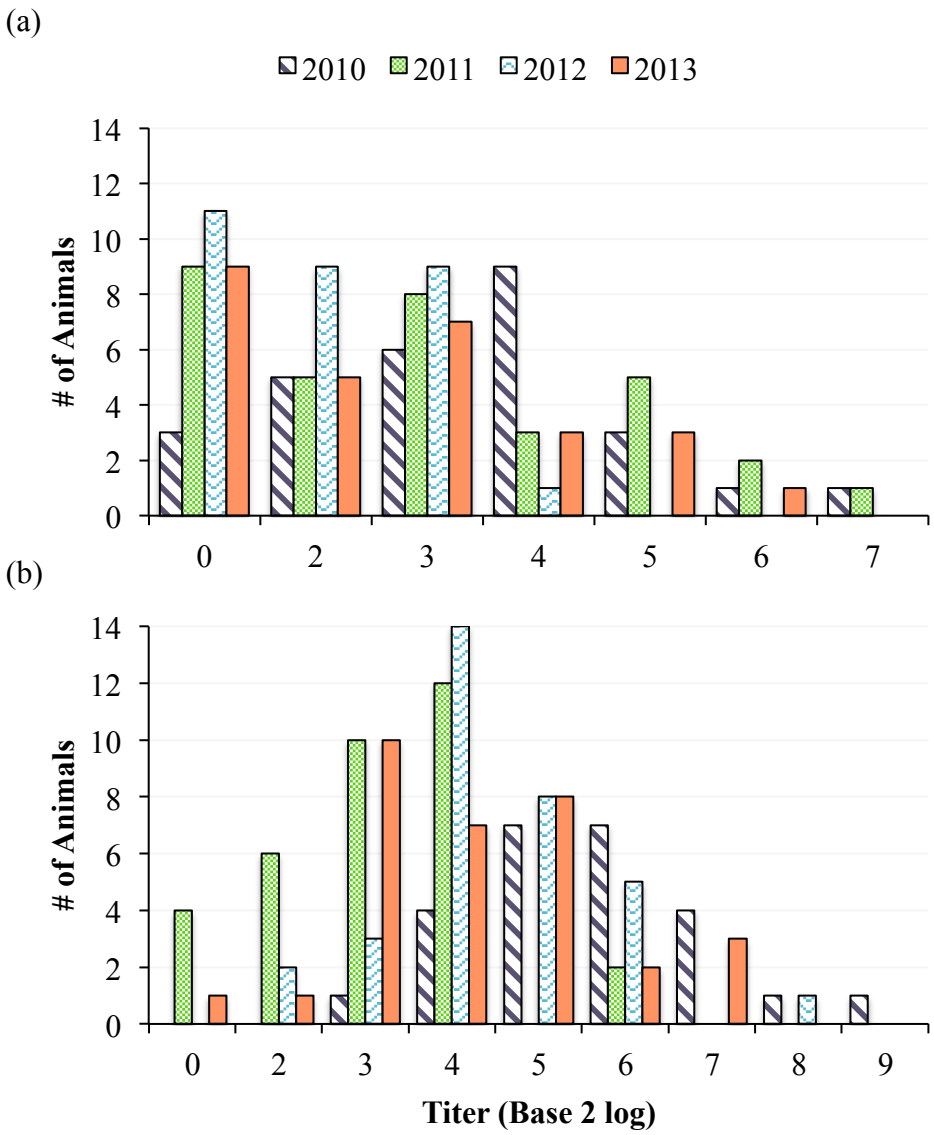


Figure 3.2. Raw Antibody Titers by Year. Distribution of antibody titers in response to vaccine treatments at day 0 (a) Killed vaccine (KV) and (b) MLV vaccine prior to challenge across 4 years.

Response to the BVDV 1b Challenge

The maximum platelet decline observed was –93.6%, while maximum lymphocyte decline was –80.6%. Animals in the control treatment group had the greatest decline in circulating lymphocytes and platelets. Lymphocyte counts in animals receiving MLV treatment declined less ($P < 0.05$) than in animals receiving either the KV or control treatments (Figure 3.3a). The average maximum platelet decline in the MLV treatment was less than in the control treatment ($P < 0.05$); the platelet decline in the KV treatment was intermediate to MLV and control, and not statistically different from either (Figure 3.3b).

The highest individual recorded temperature in the first 14 days PC was 41.8°C, in the control treatment. Mean day 0 temperatures were not different among treatments; steers receiving the MLV vaccine had lower mean rectal temperatures through day 7 PC compared to the other treatments ($P < 0.001$) (Figure 3.3b). No differences were observed among treatments for mean rectal temperatures by day 10 PC. In addition to rectal temperature collections, animals were observed daily for additional clinical respiratory symptoms. No animal met the threshold for clinical illness or warranted additional rectal temperature measurements for therapeutic treatment based on clinical symptoms.

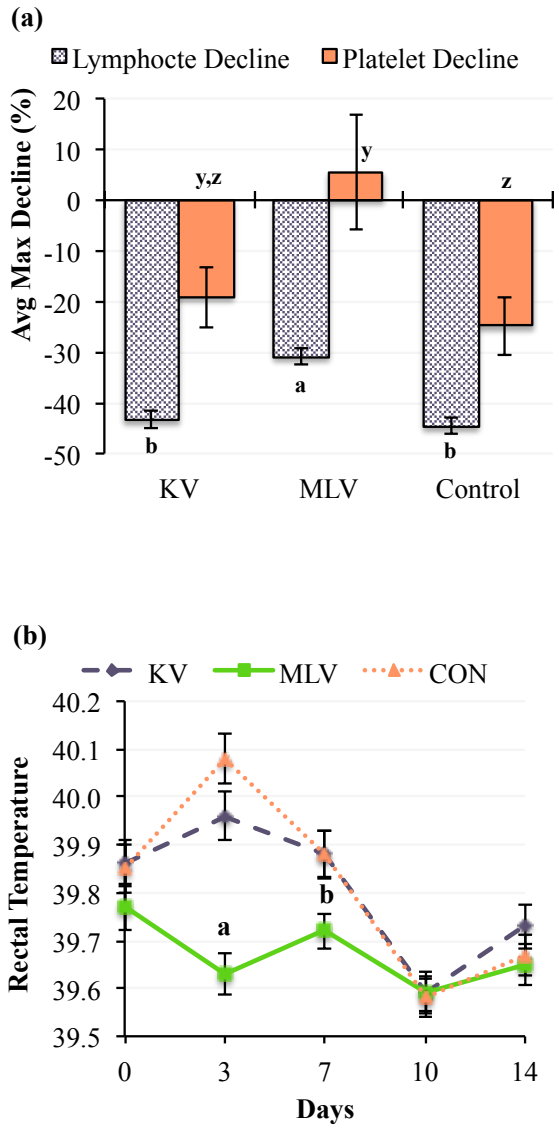


Figure 3.3. Lymphocyte and Platelet Decline and Rectal Temperature Response to BVDV Challenge. (**a**) Greatest decline (%) in circulating lymphocytes and platelets by treatment (killed vaccine (KV), modified live virus (MLV) vaccine, or control (non-vaccinated)) post-challenge with BVDV 1b CA0401186. Data displayed as means ±SEM. Superscripts[a,b] represent significant differences ($P < 0.001$) between treatment groups for lymphocyte decline. Superscripts[y,z] represent significant differences ($P < 0.05$) between treatment groups for platelet decline. (**b**) Mean rectal temperature by treatment (killed vaccine (KV), modified live virus (MLV) vaccine, or control (CON; non-vaccinated)) across the first 14 days post-challenge. Significant treatment differences within day are denoted by [a] $P < 0.001$ between MLV and KV/Control treatments, and [b] $P < 0.05$ between MLV and KV/Control treatments.

When challenged, 20.89% of the animals did not present with pyrexia or subclinical signs. Lymphopenia was the most frequent sign of subclinical disease (55.15%) PC. Thrombocytopenia was observed in 40.67% of the animals and 40.11% of the animals presented with pyrexia PC. These responses varied by treatment and year (Table 3.2).

Vaccine Effects on Response to BVDV 1b Challenge

Vaccine treatment affected the response to the challenge for most phenotypes. The MLV treatment had the greatest proportion of animals (34.68%) without pyrexia and subclinical signs PC (Table 3.2). No difference was observed in protection from the KV treatment compared to the control treatment for prevention of lymphopenia or no symptoms (Table 3.2). The MLV treatment significantly reduced ($P < 0.05$) the number of animals that presented with lymphopenia to approximately half of the KV and control treatments (Table 3.2). Thrombocytopenia was observed in significantly fewer individuals ($P < 0.05$) receiving vaccines compared to controls (Table 3.2). No treatment differences were observed in the percentage of animals that presented with pyrexia.

Table 3.2. Disease Symptoms by Treatment. Percentage of animals with (1) no disease signs (2) clinical sign of pyrexia (2 or more consecutive time points of rectal temperature > 1 SD from baseline temperature), (3) lymphopenia (>40% decline in lymphocyte counts), and (4) thrombocytopenia (>40 % reduction in platelet counts) following BVDV 1b challenge by treatment and year.

	N	No Disease Signs	Pyrexia	Lymphopenia	Thrombocytopenia
Treatment					
KV	119	16.81[b]	40.34	64.71[b]	37.82[a]
MLV	124	34.68[a]	34.68	33.87[a]	31.45[a]
Control	116	10.34[b]	45.69	68.10[b]	53.45[b]
Year					
2010	76	10.53[a]	36.84[b,c]	71.05[a]	72.37[a]
2011	100	32.00[b]	25.00[c]	52.00[b]	29.00[b]
2012	95	20.00[a]	54.74[a]	52.63[b]	28.42[b]
2013	88	18.18[a]	44.32[a,b]	47.73[b]	39.77[b]

[a,b,c] Superscripts indicate significant differences ($P < 0.05$) between treatment or year within a column.

Year Effects of Response Phenotypes to BVDV 1b Challenge

Year effects on response phenotypes were highly variable. The year effect trended similarly for lymphopenia and thrombocytopenia (Table 3.2). Lymphopenia and thrombocytopenia were most prevalent in 2010 ($P < 0.05$); approximately 70% of the animals in that year presented with reduced cell counts in response to the challenge (Table 3.2). No differences were observed in lymphopenia and thrombocytopenia prevalence in 2011, 2012, or 2013 (Table 3.2). The greatest proportion of animals to present with no disease signs was in 2011, and that same year showed the lowest number of steers presenting with pyrexia in response to the challenge but not significantly different from 2010 levels (Table 3.2). The year with the highest proportion of pyrexia presentation was not the same year with the greatest proportion of steers with lymphopenia and thrombocytopenia (Table 3.2).

Titers and Protection from of Disease

Detectable titers were found in 70.70% of the individuals without symptoms PC (Figure 3.4a). The MLV treatment group included significantly more individuals with detectable titers and no disease signs ($P < 0.05$), accounting for 53% of the healthy animals (Figure 3.4a). No difference in pyrexia frequency was observed between vaccine titers ($P > 0.05$) (Figure 3.4b). Animals with no detectable titers presented with lymphopenia and thrombocytopenia at a higher frequency than animals with detectable levels (Figure 3.4c and 3.4d). Lymphopenia was more frequent at high titers in the MLV treatment, but was more frequent at low titer in the KV

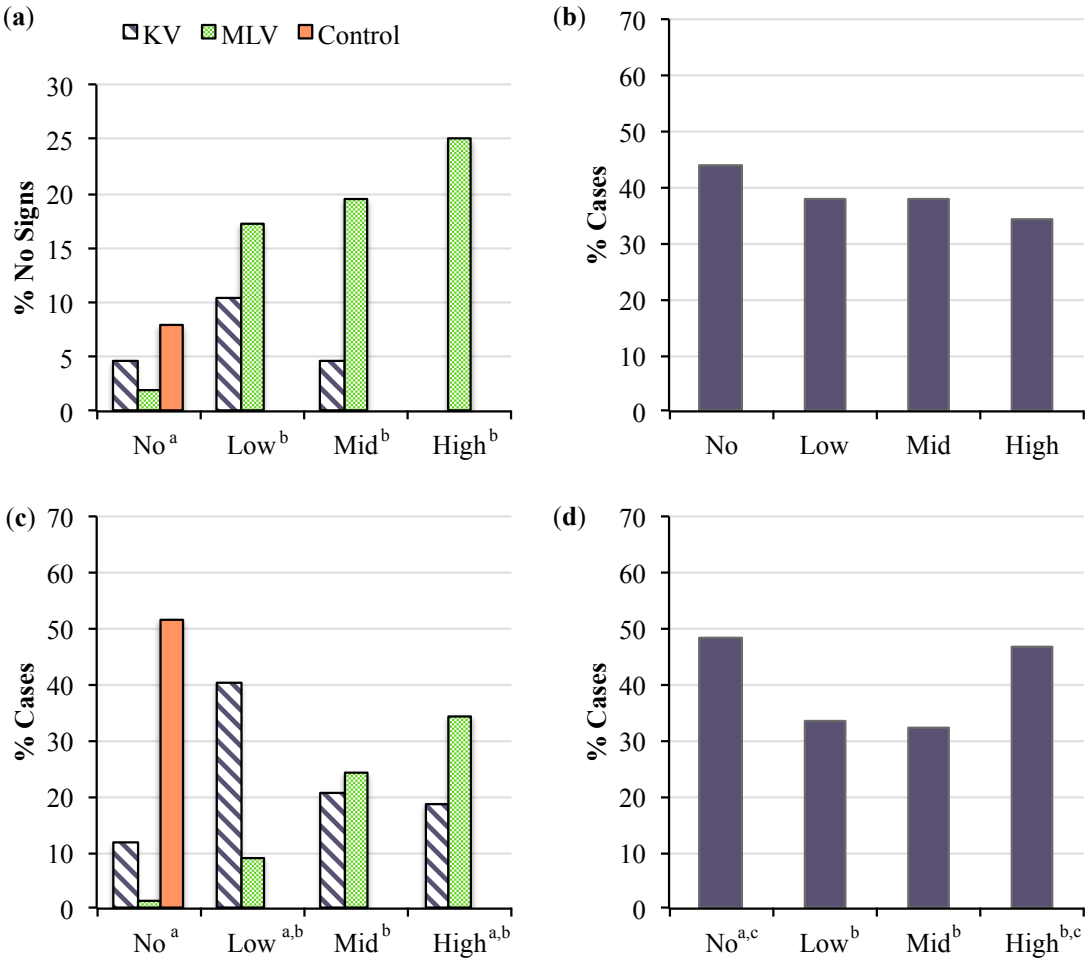


Figure 3.4. **Prevalence of Symptoms by Vaccine Titer.** Proportion of animals with no, low, mid, or high BVDV 1b vaccine titers (day 0) that present with (a) No disease symptoms, (b) Pyrexia, (c) Lymphopenia, and (d) Thrombocytopenia post BVDV 1b challenge. (No: titer = 0; Low: 2 ≤ Titer < 4; Mid: 4 ≤ Titer < 6; and High: Titer ≥ 6) that present with. [a,b,c]Alphabetic scripts represent significant ($P < 0.05$) differences between titers. (a) and (c) Had significant interactions for titers by treatment.

treatment (Figure 3.4c). When vaccine titers were in the low to mid range, the frequency of thrombocytopenia presented less frequently than when no titers were detectable (Figure 3.4d). No treatment interaction was observed between antibody titers post-vaccination and pyrexia or thrombocytopenia ($P > 0.05$).

Clinical versus Subclinical Response

The frequency of lymphopenia and thrombocytopenia with pyrexia (clinical) and without pyrexia (subclinical) were compared (Figure 3.5). The subclinical presentation occurred at 1.24 times as often as clinical presentation. Subclinical disease symptoms occurred more frequently in steers with mid to high titers. No differences in numbers of clinical vs subclinical cases were observed in steers with no to low titers (Figure 3.5).

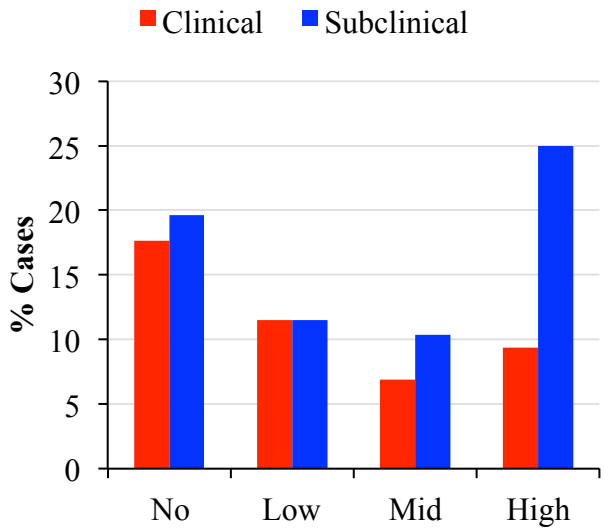


Figure 3.5. **Clinical versus Subclinical Disease by Vaccine Titer.** Frequency of animals that presented with clinical (pyrexia, lymphopenia, and thrombocytopenia) symptoms versus the subclinical (lymphopenia and thrombocytopenia) signs of disease across the four vaccine titer categories (no, low, mid, and high).

Anamnestic Antibody Response to BVDV Challenge

The greatest anamnestic antibody response was observed in the KV treatment group, by day 14 PC (Figure 3.6a). Year differences in the anamnestic response to vaccination were similar to those described for response to vaccination (data not shown). The MLV treatment had higher titers on day 0 in response to vaccination, but demonstrated lower anamnestic antibody response to the challenge than the KV treatment (Figure 3.6a). By day 42, no difference was observed between the MLV and control treatment (Figure 3.6a). Anamnestic antibody response PC was not different for healthy versus sick animals that presented with pyrexia, lymphopenia, or thrombocytopenia or the combination ($P > 0.05$) (Figure 3.6b).

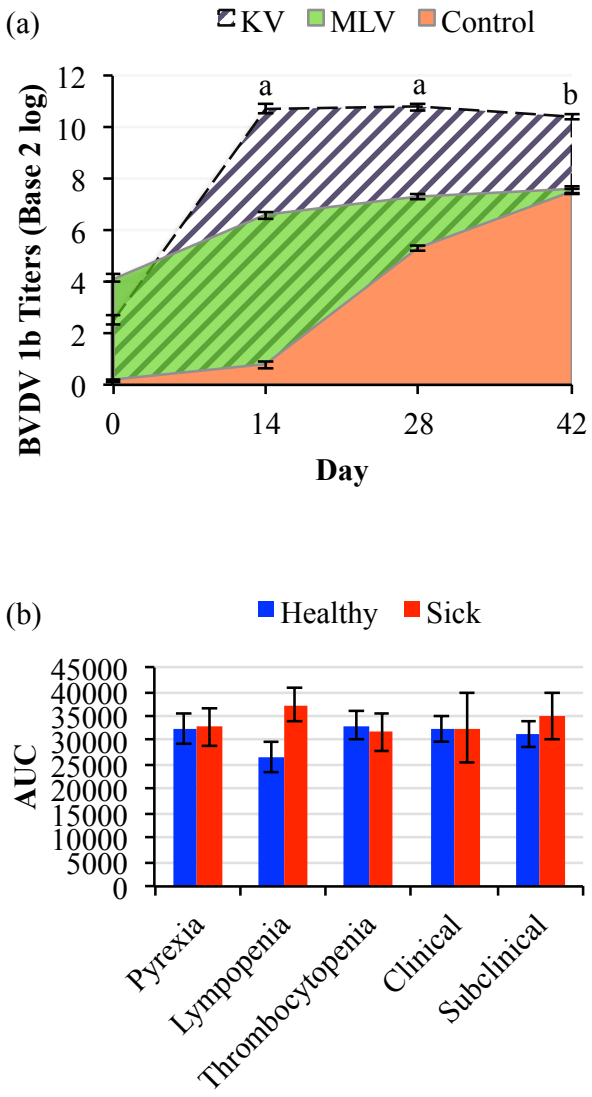


Figure 3.6. **Anamnestic Antibody Response.** (**a**) Mean antibody titer (base 2 log) for BVDV type 1b at days 0, 14, 28, and 42 with area under the curve (AUC) shaded for each vaccine treatment (killed vaccine (KV), modified live virus (MLV) vaccine, or control (non-vaccinated)). Means reported for each treatment at days 0, 14, 28 and 42 post-challenge with SEM. [a] Significant ($P < 0.05$) differences between KV, MLV, and control treatments. [b] Significant ($P < 0.05$) difference between KV and MLV/Control treatments. (**b**) Mean BVDV 1b antibody titers for healthy (animals not presenting with phenotypes of interest: pyrexia, lymphocytopenia, or thrombocytopenia) and sick (animals that present with one or more phenotypes: pyrexia, >40% reduction in circulating lymphocytes and platelets), displayed as AUC with SEMs.

Discussion

No or low detectable levels of neutralizing antibodies do not indicate lack of protection as animals with primed immune responses can be protected against pathogens as we observed in the BVDV challenge in steers with no detectable antibody (ENDSLEY *et al.* 2004; REBER *et al.* 2006). We observed that the MLV treatment elicited higher antibody titers compared to the KV treatment, although previously killed vaccines have been reported to generate higher titers (CHASE *et al.* 2003). Conversely, in anamnestic antibody response the paradigm remained true, the KV treatment elicited higher anamnestic response compared to the MLV treatment PC. But MLV vaccines are known to elicit more robust and longer lasting immune responses compared to killed vaccines, suggesting why the vaccine titers were higher for the MLV treatment (RIDPATH 2005; RIDPATH *et al.* 2010; RIDPATH 2013). Animals that were vaccinated with an MLV vaccine were better protected against the challenge, especially steers in the no, low and mid titers. Similar to our findings, vaccinated animals were reported to have protection even in the absence of titers from vaccination (STEVENS *et al.* 2011). Conversely, calves with lower serum neutralizing antibody levels at entry to the feedlot have been reported to be at increased risk for BRD treatment, illness, and reduced net value to the owner (FULTON 2015). Our results suggest that the immune response elicited by the MLV vaccine stimulated more than a humoral antibody response, as animals with no and low titers were better protected than the KV treatment animals at the same titers. Furthermore, protection against a BVDV challenge can occur in the absence of detectable post-vaccination antibody titers (RIDPATH 2003; PLATT *et al.* 2009), and

several studies have shown little correlation between increased antibody titers and disease prevention (REBER *et al.* 2006). Our results correspond with others' suggesting that the type of immune response may be more important for protection against viral pathogens than increased titers of neutralizing antibodies (CHASE *et al.* 2003; SILFLOW *et al.* 2005; CHASE 2013): both a primed T- and B-cell response may be required to offer optimal protection against the challenge.

The year effects observed were inconsistent and not identical across all treatments and phenotypes. The years with lowest antibody titers for either vaccine treatment were not those with greatest numbers of individuals with lymphopenia or thrombocytopenia. Similarly, BRD prevalence was reported to vary by year, as was observed with the prevalence and robustness of the BVDV phenotypes in these studies (SNOWDER *et al.* 2006; NEIBERGS *et al.* 2011). This may suggest that there may be a cyclical pattern in disease that is not related to the protective response from the vaccination, or it may reflect some year-to-year variation in the genetic background of the steers. The host genetic background may help explain some of the year effects (COLLEN *et al.* 2002), but further investigations are required to understand the genetic component that might influence year-to-year variation in vaccine protection. Alternatively, the year-to-year variation may be explained by unmeasured environmental exposures.

Respiratory infection in feedlots is typically diagnosed through observed clinical symptoms, and affected animals are treated based on elevated rectal temperature (DUFF

AND GALYEAN 2007a). However, multiple studies have documented no observable clinical symptoms post BVDV challenge, although leukopenia and pyrexia were reported shortly following challenge (BEER *et al.* 1997; LIEBLER-TENORIO *et al.* 2003a; RIDPATH 2003; KELLING *et al.* 2007; LIANG *et al.* 2008). These findings suggest that not all BVDV infections present with visible symptoms.

Rectal temperatures were recorded on pre-determined days rather than as a final clinical threshold following initial observation of clinical symptoms, as would be the case in a field protocol. Observations of pyrexia were observed in cattle with leukopenia similar to previous studies (CORTESE *et al.* 1998; LIEBLER-TENORIO *et al.* 2003a; ZIMMERMAN *et al.* 2006; KELLING *et al.* 2007; LIANG *et al.* 2008), but not all cattle with lymphocytopenia and thrombocytopenia experienced pyrexia. Based on our data, rectal temperature is not a reliable indicator of morbidity, and utilization of rectal temperature to identify sick cattle may miss a portion of sub-clinically ill cattle. The presence of sub-clinically ill, undiagnosed cattle in a population may have large financial impacts, which are difficult for operators to directly assess (MARTIN *et al.* 1980; MARTIN *et al.* 1981). Assessment of disease presentation by leukopenia and thrombocytopenia may increase the detection of "sick" animals compared to traditional observational diagnoses.

Lymphocytopenia and thrombocytopenia are subclinical indicators of BVDV infection. Lymphopenia was observed in both vaccinated and unvaccinated calves post-challenge, with an average reduction of 39.4% in lymphocytes following the challenge, similar to

other reports (CORAPI *et al.* 1989; LIEBLER-TENORIO *et al.* 2003b; LIEBLER-TENORIO *et al.* 2003a; RIDPATH *et al.* 2007; CHASE 2013; PALOMARES *et al.* 2014a). Large variations in circulating lymphocyte counts among steers were observed, including calves with lymphocytes elevated by 40% from baseline to calves with severe lymphopenia of 80% reductions, suggesting that individual variation exists within the population and that some calves develop a more robust immunity for protection against the BVDV challenge than others. Results from this study and others suggest that a protective response from vaccination should prevent leukopenia and thrombocytopenia during pathogen exposure (BROCK *et al.* 2007; PLATT *et al.* 2009).

Vaccination reduced the subclinical effects from the BVDV challenge. Calves that received MLV were less susceptible to lymphopenia and thrombocytopenia than non-vaccinated animals, similar to other studies (LIANG *et al.* 2006; KELLING *et al.* 2007; RIDPATH *et al.* 2010). The MLV treatment appeared to elicit a more robust immunological response to protect against lymphopenia compared to the KV treatment, although unmeasured. Vaccinated steers displayed less severe lymphocytopenia and thrombocytopenia following the challenge than unvaccinated steers, and this concept is likely important in production environments. The lower antibody titers observed in the anamnestic response indicated that the MLV treatment had greater protection from vaccination, which resulted in less viral replication PC. This suggests that the MLV vaccine likely stimulated both a humoral and cell-mediated immune response. Titers

following pathogen exposure cannot be used to gauge the level of protection within a population.

In conclusion, this study suggests that antibody titer may not be a reliable metric of protective immune response against a BVDV 1b challenge or of disease status. Reber *et al*. (REBER *et al.* 2006) similarly reported weak to no correlations between humoral and cellular immune responses, and Ridpath (RIDPATH 2003) previously suggested that antibody titers may not be a reliable indicator of protection. The titer threshold for protection may be dependent on more than detectable humoral immune response. Based on these results the titer threshold for protection is variable and is dependent on the other immune components that have been primed by the vaccination. To find the protection titer threshold it may be critical to understand the type of vaccination and the humoral immune response stimulated, as well as animal-to-animal variation, i.e. genetics. More steers in this experiment had lymphocytopenia and thrombocytopenia than pyrexia, suggesting that rectal temperature does not identify all animals with infection. Additionally, rectal temperature used alone as a predictor for morbidity likely misses a substantial proportion of cattle with BVDV infection. These results show a benefit from BVDV vaccination even in the presence of low detectable titers, and that the MLV vaccine provided better protection against BVDV 1b challenge in a production setting. Undiagnosed, sub-clinically ill animals in commercial feedlots likely present significant health management obstacles as viral reservoirs and potential sources of reduced production efficiency. Vaccine efficacy for commercial use needs to be evaluated on the

prevention of subclinical disease presentation and the stimulation of both humoral and cell-mediated arms of the immune response. Antibodies titers alone do not appear to be indicative of the level of protection offered by a vaccination.

CHAPTER IV

VARIATION IN HOST IMMUNE RESPONSE TO BVDV VACCINATION ACROSS BOVINE SUBSPECIES

Synopsis

Vaccination is used as an industry-wide practice for control of bovine respiratory disease complex, with mixed results. The roots of vaccine failure are many with one being genetic predisposition to immune responsiveness. Differences between host immunity to vaccination in *Bos indicus* and *Bos taurus* could impact vaccine efficacy. This study aimed to identify differences between indicine and taurine cattle in immune responses to commercially available vaccines for respiratory disease, using bovine viral diarrhea as an indicator for measurement of response. Calves in a classic production setting were immunized with either killed or modified live virus (MLV) vaccinations. Blood leukocyte profiles and humoral and cell-mediated immune responses were evaluated for changes in response to vaccination. Indicine cattle were found to have larger baseline leukocyte populations of total white blood cells, which were highly influenced by the 2-fold difference in neutrophils found in Brahman calves. A unique platelet response was observed in indicine cattle, with a 50% reduction of platelets 3 days post-vaccination followed by a baseline level recovery. While indicine cattle had more total neutrophils, the taurine cattle had approximately 50% greater neutrophil responses to the vaccines that were faster, by day 3, compared to the steady neutrophil levels observed in indicine cattle. The indicine cattle appear to be more susceptible to immunosuppression in

response to the MLV vaccine, but when vaccinated with a killed virus and boosted with an MLV, the immunosuppression was mitigated.

Introduction

Infection with viral and/or bacterial pathogens has been associated with the development of bovine respiratory disease complex (BRDC). In particular, infection with bovine viral diarrhea virus (BVDV) is often suspected as an initiating event for BRDC (KELLING *et al.* 2007). Consequently, resistance to BVDV might translate into relative resistance to BRDC (RIDPATH 2013) and for this reason most of the commercially available vaccines against BRDC contain a BVDV component.

BVDV is a pestivirus (*Flaviridae)* with a positive-sense single-stranded RNA genome of ~12.5 Kb (RIDPATH *et al.* 1994; CHARLESTON *et al.* 2002; LIANG *et al.* 2008). BVDV isolates are highly heterogeneous (RIDPATH 2005) and exist as cytopathic and noncytopathic biotypes (RIDPATH *et al.* 1994; PETERHANS *et al.* 2003; CHASE 2013). Variation in the 5' untranslated region of the viral genome generates two genotypes, BVDV types 1 and 2 (RIDPATH *et al.* 1994). Pestiviruses infect both beef and dairy cattle, resulting in economic losses for operations, and risks to other ruminant livestock and wildlife (RIDPATH 2015).

Vaccination is a valuable practice used by the industry to promote animal health at all ages through prevention and control against viral and bacterial pathogen outbreaks

(DUFF AND GALYEAN 2007a) and has been used as the primary prevention method for BVDV infections. However, widespread vaccination has not significantly reduced the incidence of BRDC (GLASS 2004). Host variation in immune response (CORTESE *et al.* 1998; GLASS 2004; FULTON 2005) and evasion mechanisms of the pathogen can reduce the effectiveness of vaccination (SRIKUMARAN *et al.* 2007; RIDPATH 2013). Most vaccine efficacy studies have been done in taurine cattle while many cattle raised in the southern regions of the United States are crosses of indicine and taurine cattle. In this study, we asked whether variation exists between indicine and taurine cattle in response to BVDV killed and modified live vaccines.

Host variation is known to play a critical role in antigen recognition and lymphocyte proliferation (BROCK *et al.* 2007; SRIKUMARAN *et al.* 2007; ABBAS *et al.* 2011). Several studies conducted primarily in taurine breeds demonstrate variation in vaccination response among individuals (RIDPATH *et al.* 2010; BAUERMANN *et al.* 2013; RICHESON *et al.* 2013a); however, vaccine effectiveness and individual variation in immunization against BVDV have not been well studied in indicine cattle.

Both humoral and cell-mediated immunity afford protection against respiratory infection (BROCK *et al.* 2007; MACEDO *et al.* 2013; RIDPATH 2013), and the evaluation of efficacious vaccination needs to interrogate both cell-mediated and humoral responses (REBER *et al.* 2006). Typically, humoral response is most often used to measure the response to vaccination due to the relative ease in measuring humoral response

compared to cell-mediated response. However, both humoral and cell-mediated responses have been studied in animals vaccinated with either killed or MLV products. The level of humoral protection produced by the two different types of vaccines varies, along with the ratio of B:T-cell activation (SANDBULTE AND ROTH 2003; STEVENS *et al.* 2009). Furthermore, protective immunity can be established in the absence of detectable antibody levels (CORTESE *et al.* 1998; FULTON *et al.* 2000; DESCÔTEAUX *et al.* 2003) and, conversely, protection against viral challenges has been shown in the absence of detectable proliferating T-cells (CORTESE *et al.* 1998; FULTON *et al.* 2000; ELLIS *et al.* 2001; ENDSLEY *et al.* 2003).

Differences in cellular and humoral immunity between *Bos indicus* and *Bos taurus* cattle may extend to individualized disease resistance (MACEDO *et al.* 2013). BVDV has been shown to affect both the function of the innate immunity and the stimulation of the adaptive immune response (PETERHANS *et al.* 2003). BVDV causes immunosuppression of the adaptive immune system (CHASE 2013; RIDPATH 2013), but few studies have examined the differences in the immunosuppression between indicine and taurine breeds.

The objectives of this study were to test for differences in response to vaccination based on leukocyte profiles and cell-mediated and humoral immune responses between *Bos indicus* (Brahman) and *Bos taurus* (Charolais) calves in response to commercially available vaccines in a production setting.

Material and Methods

This project was reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee and the Texas A&M University Institutional Biosafety Committee.

Animal Population and Treatment

Fifteen purebred Brahman (*Bos indicus*) and 15 purebred Charolais (*Bos taurus*) calves, 3 to 6 months of age, with no previous vaccination history, were used for this trial. Calves were bulls or heifers and housed on a local ranch in eastern central Texas. Calves were tested for BVDV persistent infection at the Texas Veterinary Medical Diagnostics Laboratory (TVMDL; Amarillo, TX) prior to enrollment using antigen-capture ELISA assay on ear notch samples. All calves were confirmed free of persistent infection before enrollment in the study. Calves were randomly assigned to 1 of 5 vaccine treatments, with 3 calves of each breed per treatment for a total of 6 calves per treatment. Two-shot protocols were followed for all treatments: initial vaccination was given on day 0 followed by a booster vaccination on day 21, administered subcutaneously according to label directions. The five treatments were: (1) **control** - no vaccination or sham injection; (2) Adjuvant control (**ADJ**) - Notavaris ReproSTAR® VL5HB (Novartis Animal Health US, Inc., Greensboro, NC), which contains the adjuvant Xtend®SP and protects against *Camplobacter fetus*, *Leptospira canicola*, *L. grippotyphosa*, *L. hardjo-bovis*, *L. icterohaemorrhagiae*, and *L. pomona* but does not have a BVDV component; (3) modified live virus (**MLV**) vaccine – Novartis BRD Shield^{TM}, licensed for the

prevention of infectious bovine rhinotracheitis, BVDV types 1 and 2, parainfluenza type 3, and bovine respiratory syncytial virus; (4) killed vaccine (**KV**) - Novartis Vira Shield ® 6, which contains the adjuvant Xtend®SP and is licensed for prevention of the same pathogens as the MLV product; and (5) a single dose of Vira Shield ® 6, followed by a single dose of BRD Shield™ (**MIX**). New, sterile needles and syringes were used for each calf.

Peripheral Mononuclear Blood Cell (PMBC)

Forty milliliters of whole blood for PBMC isolation was collected at the initial vaccination (day 0, baseline), then on days 7, 14, 21 (booster vaccination), 28, 35, and 42 post first vaccination. Jugular blood was collected in 60 mL syringes with 8 mL acid citrate dextrose. PBMCs were isolated by separation over Ficoll-Paque™ Plus (GE Healthcare, Marlborough, MA), washed in Alsievers to remove platelets, and cell pellets were resuspended in RPMI 1640 with 10% fetal bovine serum, 1% glutamax, 0.025% Hepes, 0.01% gentimycin, and 0.01% beta-mercaptoethanol (RPMI 1640 Complete).

IFN-γ EliSpot Assay

MultiScreen-IP filter plates (EMD Millipore, Darmstadt, Germany) were coated overnight with mouse anti-bovine IFN-γ monoclonal antibody at 4°C. Plates were washed with PBS and blocked with 2% bovine serum albumin-PBS for 2 hours at 37°C. PBMCs were dispensed at 2.5 x 10^5 cells per well in triplicate complete RPMI 1640 media, 5 µg/mL Con-A control, or 2.5 µg/mL BVDV peptide mix (COLLEN *et al.* 2002)

and incubated at 37°C for 12 hours overnight. IFN-γ-secreting cells were detected with biotinylated antibody (5 μg/mL in 2% BSA-PBS) as spots on the membrane and visualized with a Vectastain PBC peroxidase substrate (Vector Labs, Burlingame, CA). Plates were dried overnight and spots were counted on plate reader with Elispot 3.4 software (AID GmbH, Strassberg, Germany). The number of cells secreting IFN-γ was expressed as the difference between the average number of spots in the BVDV peptide stimulated wells and the average of the media control wells (both done in triplicate).

T-Cell Proliferation

BVDV-specific T-cell proliferation was measured in PBMCs using a thymidine-uptake assay. PMBCs were added at a concentration of 2.5 x 10^5 cells per well in triplicate and were stimulated *in vitro* with either complete RMPI 1640 media alone or with 5 μg/mL BVDV peptide mix (COLLEN *et al.* 2002). Cells were cultured for 48 hours at 37°C with 5% CO_2, then pulsed with 0.25 μCi thymidine per well and harvested 18 hours later with a Tomtec Harvester, MACH III M (Triad Scientific, Manasquan, NJ). Thymidine uptake was measured on a Microbeta Trilux, 1450 LSC & luminescence counter (Perkin Elmer, Waltham, MA). Proliferation response was measured as the average of the triplicate wells and expressed as the difference between the BVDV stimulated proliferation to the media proliferation.

Serum Collection

Jugular blood was collected in BD Vacutainer® serum tubes (Becton Dickinson, Franklin Lakes, NJ) on days 0, 3, 7, 14, 21, 24, 28, 35 and 42 post first vaccination. Samples were kept at 4°C overnight, brought to room temperature, centrifuged, sera removed and stored at -20°C until assayed.

Viral Neutralization Assays

Viral neutralization assays were performed on sera collected on days 0, 7, 14, 21, 28, 35, and 42 post first vaccination. Viral neutralizations of BVDV type 1a Singer and BVDV type 2 296c were performed in 5 replicates as described (DOWNEY *et al.* 2013). Neutralizing antibody titers were reported as the base 2 log of the highest dilution that showed no cytopathic effect.

Hematology

Differential white blood cell counts (WBC), platelets, and red blood cell (RBC) counts were determined on a CELL-DYN 3700 blood analyzer (Abbott Laboratories, Abbott Park, IL) at the University of Arkansas Nutrition Laboratory (Fayetteville, AR) on whole blood collected in 0.2 μM EDTA tubes on days 0, 3, 7, 14, 21, 24, 28, 35, and 42 post-vaccination.

Cortisol

Cortisol concentrations were determined using a single antibody radioimmunoassay (MP Biomedicals, LLC Diagnostic Division, Orangeburg, NY) on serum samples from days 0, 3, 7, 14, 21, 24, 28, 35, and 42 days post first vaccination. The antibody radioimmunoassay utilized rabbit anti-cortisol antiserum coated tubes according to manufacturer's instruction. The lowest detectable serum concentration was 0.25 ng/mL. A standard curve was generated by plotting the results generated from standard samples with know cortisol concentrations. Cortisol concentrations (ng/mL) were determined by comparison to a standard curve that was generated each time samples were analyzed.

Data Analysis

Blood leukocyte data analysis was performed using the percent change relative to the day of first or second vaccination. The response to the first vaccination (vaccination first dose **V1D**) was defined as the difference between values on day 0 (prevaccination baseline) and values on days between day 0 and day 21, and the response to the second vaccination (vaccination second dose or **V2D**) was defined as the difference between values on day 21 (day of booster vaccination) and values on days after day 21. Percentage change for both responses was calculated as the difference from days 3, 7, and 14 post-vaccination (days 3, 7 and 14 for first vaccination and days 24, 28 and 35 for second vaccination), relative to the day of vaccination (day 0 for first vaccination or 21 for second vaccination). Treatment means were an average of the 3 individuals per breed within a treatment. Treatment effects were evaluated using a 2-tailed *t*-test with equal

variance, comparing the mean of the treatment percent change to the mean of the control treatment percent change for all compared cell types. Breed differences in percent change on a specific day were evaluated using a 2-tailed *t*-test with equal variance. We used a 2-tailed paired *t*-test to analyze differences between the V1D and V2D responses measured within calves of the same treatment at days 3, 7, and 14 post initial and booster vaccination. Two-way ANOVAs with repeated measures when necessary were performed in GraphPad Prism 6.0 (San Diego, CA) on triplicate means for treatment groups for EliSpot and proliferation assays.

Results

Breed Differences in Response to Vaccination

Indicine calves had higher WBC, neutrophil, RBC, and platelet counts than taurine calves prior to vaccination (Figure 4.1). Mean total white blood cell count prior to vaccination was 41% greater ($P < 0.05$) in indicine than in taurine calves (Table 4.1; Figure 4.1a). A 2-fold greater abundance of neutrophils was the major contributor to the increased number of WBC in indicine compared to taurine calves (Table 4.1; Figure 4.1c). The indicine calves also had approximately 10% more RBCs compared to the taurine calves (Table 4.1). The increased blood leukocyte profile extended to platelet counts as well; the indicine calves had 80% more platelets on day 0 (Table 4.1; Figure 4.1h). Serum cortisol concentrations on day 0 were significantly higher in indicine than in taurine calves (Table 4.1; Figure 4.2a) and remained higher though day 35; by day 42 no difference was observed between the breeds (Figure 4.2b). More individual variation

for eosinophils, basophils, RBC, platelets, and cortisol concentrations was observed in baseline counts for the indicine cattle than for taurine cattle.

A breed difference in response was observed for the platelet profile 3 days post VD1 and VD2; the platelet response was breed-specific, not treatment-specific. Changes in platelets in the first 3 days following the vaccinations (day 3 and 24) were the only breed differences observed in the ADJ treatment (Table 4.2). Indicine calves had marked reduction in platelets: 56% and 68% reduction at 3 days after V1D and V2D, respectively. The taurine calves exhibited minor changes, a 3% increase or a 3 % decrease respectively, following first and second vaccinations (Figure 4.3). A similar breed affect was observed, with varying degrees of changes, in all treatments (Figure 4.4).

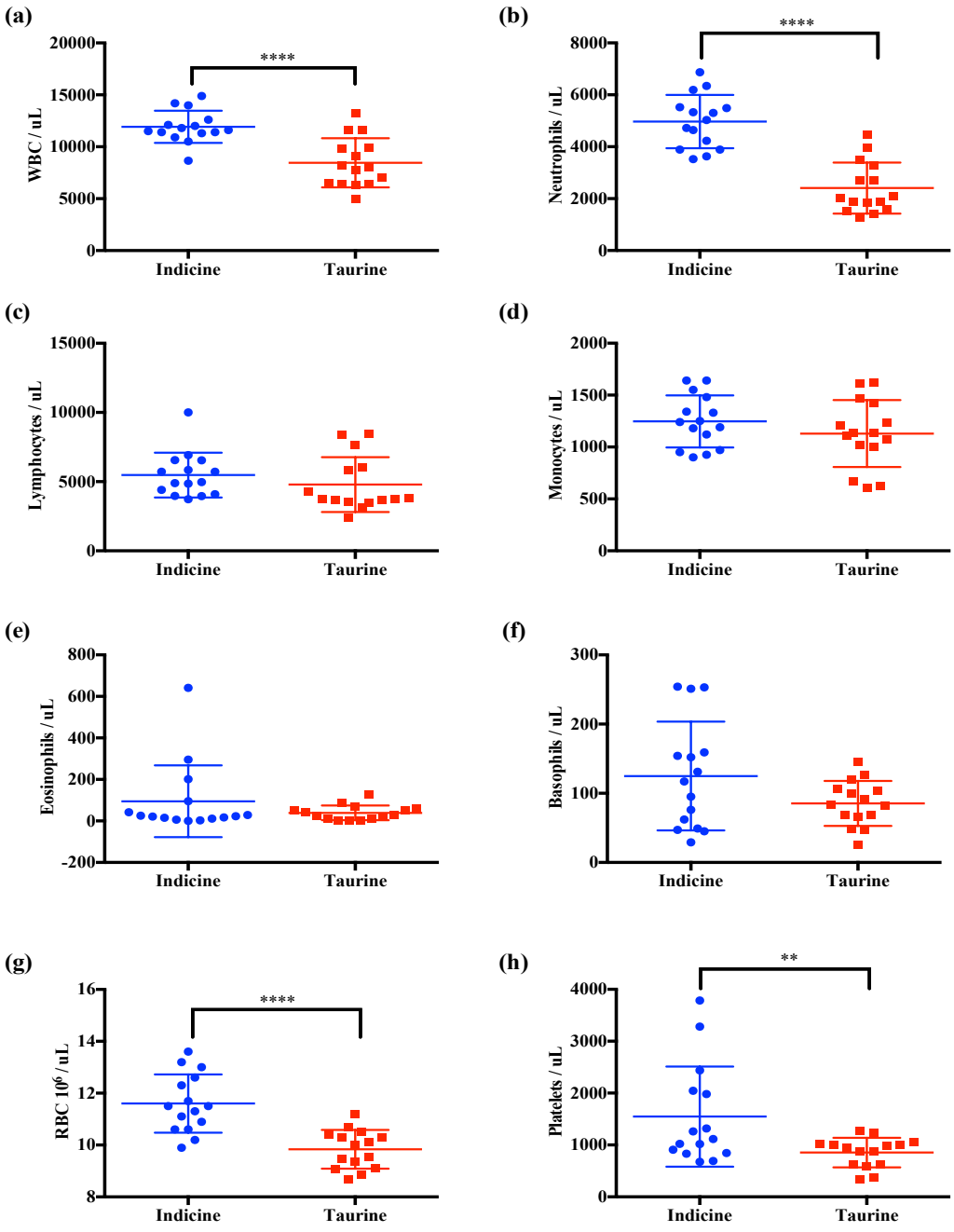


Figure 4.1. **Baseline Leukocyte Profiles.** Baseline (day 0) leukocyte absolute cell counts for Indicine (Blue; n = 15) and Taurine (Red; n = 15) cattle. (a) White blood cells (WBC); (b) Lymphocytes; (c) Neutrophils; (d) Eosinophils; (e) Monocytes; (f) Basophils; (g) Red blood cells (RBC); and (h) Platelets. Differences in baseline levels are noted by ** = $P < 0.05$ and **** = $P < 0.0001$.

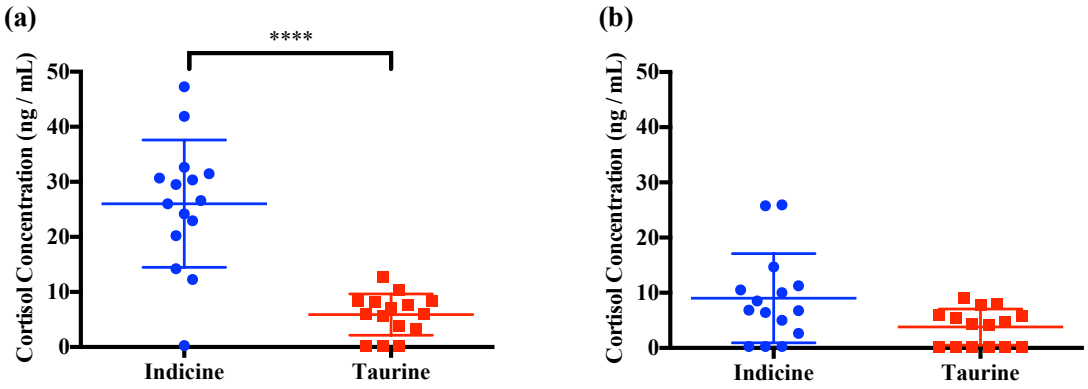


Figure 4.2. **Baseline Cortisol Concentrations. (a)** Baseline (day 0) cortisol concentrations and **(b)** Day 42 cortisol concentrations for Indicine (Blue; n = 15) and Taurine (Red; n = 15) cattle. Differences in baseline concentrations are noted by **** = $P < 0.0001$.

Table 4.1. Baseline Leukocyte Profile. Differences in baseline levels of blood leukocyte populations and serum cortisol concentrations between Indicine cattle (n = 15) and Taurine cattle (n = 15).

Phenotype	Significant Breed Difference[1] ($P < 0.05$)	Direction of Breed Difference[2]	Proportion of Difference[3]
White Blood Cells	Yes	Indicine > Taurine	0.41 Fold Greater
Lymphocytes	No	Indicine = Taurine	
Neutrophils	Yes	Indicine > Taurine	1.10 Fold Greater
Eosinophils	No	Indicine = Taurine	
Monocytes	No	Indicine = Taurine	
Basophils	No	Indicine = Taurine	
Red Blood Cells	Yes	Indicine > Taurine	0.08 Fold Greater
Platelets	Yes	Indicine > Taurine	0.81 Fold Greater
Cortisol	Yes	Indicine > Taurine	3.41 Fold Greater

[1] Significant difference of $P < 0.05$ between Indicine cattle and Taurine cattle cell counts on day 0

[2] Breed difference notates direction of the significant difference, the breed with the larger baseline level

[3] Proportion of difference between the two breeds at baseline (day 0) when significantly difference at $P < 0.05$

A breed difference in neutrophil response was observed in all 3 vaccine treatments: KV, MLV, and MIX (Tables 4.3, 4.4, and 4.5). Pre-vaccination neutrophil counts were higher and generally remained more stable in Indicine calves than in Taurine calves for all vaccine treatments. At 14 days after V1D with a MLV, taurine cattle had a 13% reduction in neutrophils, compared to a 45% reduction in neutrophils for indicine cattle (Figure 4.5a). At 3 days after V1D with a KV, an indirect relationship in neutrophil response was observed between Taurine and Indicine cattle, with a 100% increase in neutrophils in taurine calves compared to a 13% decrease in neutrophils in indicine calves (Figure 4.5c). A similar response was observed in the single dose of a KV in the MIX treatment (Figure 4.5e).

Trends in neutrophil change following the V2D were different between breeds and were dependent on the type of initial vaccination received. The trend in the V2D neutrophil response to the MLV booster vaccination was higher by approximately 50% in the MIX treatment for both breeds at day 35 compared to the V2D MLV response (Figure 4.5f). Taurine calves had a sustained 50% increase in neutrophils V2D following a MLV booster in the MIX treatment ($P < 0.05$); the neutrophil change at day 3 in taurine calves was similar to the V2D response observed in the KV treatment but was sustained for 14 days longer (Figures 4.5d and 4.5f). The Indicine calves had comparable neutrophil changes in the MLV and MIX treatment through day 28 but had lower neutrophil change by approximately 55% in the MIX treatment through day 14 compared to the Taurine

calves ($P < 0.05$) (Figure 4.5f). At day 35, V2D in the MIX treatment breed differences in neutrophil response disappeared (Figure 4.5f).

The taurine calves had approximately 25% less reduction in monocytes at day 3 after V2D than the Indicine calves in the MLV treatment (Table 4.3; Figure 4.6b). Similar breed effects were observed for lymphocyte changes in the KV and MLV treatments (Tables 4.3 and 4.4). Indicine cattle had a 5-fold increase in lymphocytes at 14 days post V1D for both KV and MLV treatments (Figures 4.7a and 4.7c). Taurine calves had a 20% greater increase of WBCs within 3 days of the V2D (day 24) with the MLV booster compared to taurine calves in the MIX treatment (Table 4.5; Figure 4.8f).

Table 4.2. **Adjuvant Treatment Response Effects on Leukocyte Profile.** Effects of adjuvant on percentage change in blood leukocytes in Indicine (n = 3) and Taurine (n = 3) at vaccination first dose (V1D; day 0-3) and vaccination second dose (V2D; day 21-24).

Phenotype	Treatment Difference[1] ($P < 0.05$)	Breed Difference[2]		Proportion of Difference[3]	
		V1D	V2D	Indicine	Taurine
White Blood Cells	No	No	No		
Lymphocytes	No	No	No		
Neutrophils	No	No	No		
Eosinophils	No	No	No		
Monocytes	No	No	No		
Basophils	No	No	No		
Red Blood Cells	No	No	No		
Platelets	No	0.07	0.03	-56.4% -68.5%	3.74% -3.04%

[1] Significant difference ($P < 0.05$) in percentage change in cell profile between adjuvant treatment and the control treatment

[2] Significant difference in percentage change in blood cells between Indicine and Taurine cattle at vaccination first dose (V1D) and vaccination second dose (V2D), *P*-value shown when approaching ($P < 0.1$) or significant ($P < 0.05$).

[3] Percentage change by breed, for cell profiles with significant differences in percentage change between the breeds. Response to vaccination first dose (V1D) percentage change listed first followed by percentage change in the response to the vaccination second dose (V2D).

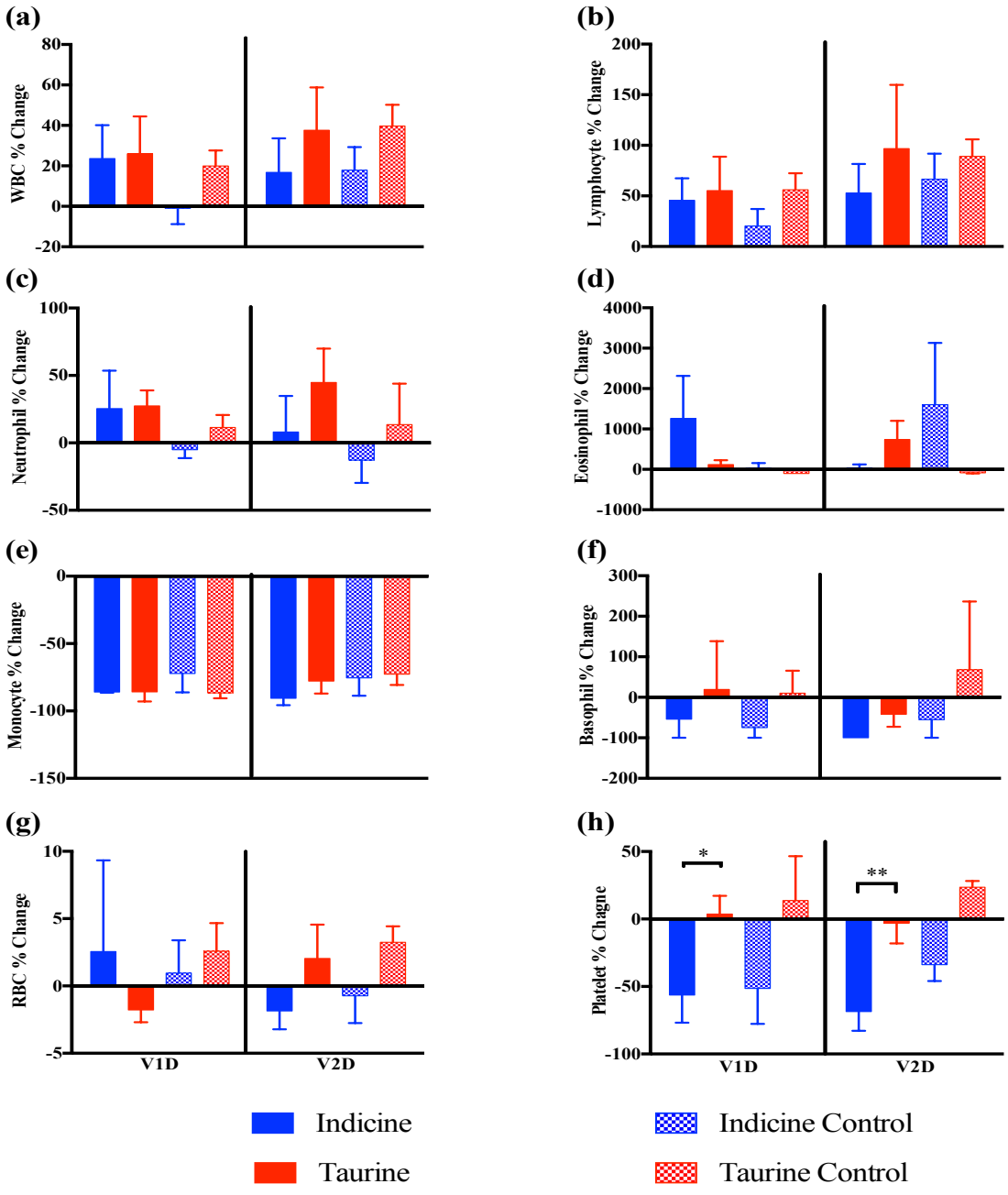


Figure 4.3. **Leukocyte Profile Response to Adjuvant.** Adjuvant-induced changes in blood leukocytes. Percentage change for the vaccination first dose response (V1D; day 0-3) and vaccination second dose response (V2D; day 21-24) is shown for the adjuvant treatment Indicine (blue, n = 3) and Taurine (red, n = 3) and for the Indicine control (blue shaded, n = 3) and Taurine control (red shaded, n = 3). Date are displayed as breed mean percent change with SEM. Significant differences are noted * $P < 0.1$ and ** $P < 0.05$.

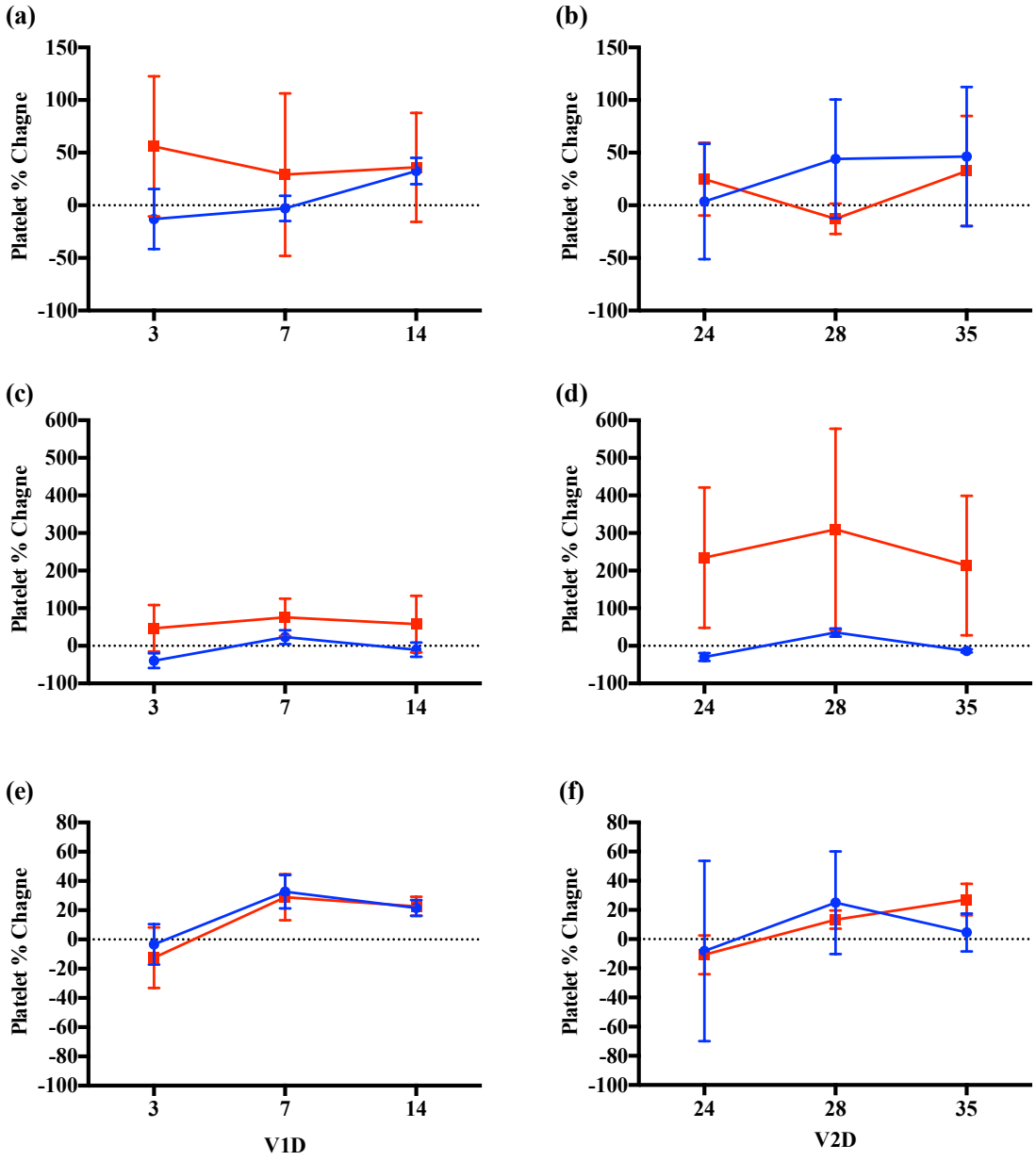


Figure 4.4. Platelet Response to Vaccination Treatment. Percent change in Platelets for vaccination first dose (V1D; days 0-14) and vaccination second dose (V2D; days 21-35) responses for MLV, killed vaccine (KV), and MIX treatments: (a) MLV V1D; (b) MLV V2D; (c) KV V1D; (d) KV V1D; (e) MIX V1D; (f) MIX V2D. Percentage change is calculated from day 0 for the V1D response and from day 21 in the V2D response. Data are displayed as mean percentage change with SEM for Indicine (blue, n = 3) and Taurine (red, n = 3). Black * = significant ($P < 0.05$) breed difference; Red/Blue * = Difference between V1D and V2D responses within a breed.

Table 4.3. **MLV Treatment Effects.** Effects of modified live virus (MLV) vaccinations on percentage change in leukocyte cell profiles in Indicine (n = 3) and Taurine (n = 3) calves at vaccination first dose (V1D; day 0-14) and vaccination second dose (V2D; day 21-35) responses.

Phenotype	Treatment Effect[1]		Breed Difference[2]	V1D vs V2D[3]	
	Indicine	Taurine	Significant Effect	Indicine	Taurine
White Blood Cells	Increase	No	No	No	No
Lymphocytes	Increase	No	Indicine > Taurine	No	No
Neutrophils	Decrease*	Decrease*	Indicine < Taurine*	No	1 < 2
Eosinophils	No	Decrease*	No	No	No
Monocytes	Decrease	Decrease	Indicine < Taurine	No	No
Basophils	Decrease	No	No	No	No
Red Blood Cells	No	No	No	No	1 > 2
Platelets	No	No	No	No	No

[1] Significant difference ($P < 0.05$) or * approaching significance ($P < 0.10$) between percentage change in leukocyte cell type between MLV treatment and control treatment for Indicine and Taurine cattle, significant treatment effects are noted by the direction of percentage change in the MLV treatment for each breed respective to the control treatment.

[2] Significant difference ($P < 0.05$) in percentage change between Indicine and Taurine cattle at any time point. Cell types with significant differences are noted with direction of difference between breeds.

[3] V1D versus V2D is differences ($P < 0.05$) in the percentage change between the vaccination first dose (V1D; days 0-14) and the vaccination second dose (V2D; days 21-35) responses within a breed. Significant differences between V1D and V2D responses are noted with the direction of difference between the two time points.

Table 4.4. Killed Vaccine Treatment Effects. Effects of killed vaccine (KV) treatment effects on percentage change in blood leukocyte cell profiles in Indicine (n = 3) and Taurine (n = 3) calves at vaccination first dose (V1D; day 0-14) and vaccination second dose (V2D; day 21-35) responses.

Phenotype	Treatment Effect[1]		Breed Difference[2]	V1D vs V2D[3]	
	Indicine	Taurine	Significant Effect	Indicine	Taurine
White Blood Cells	Increase	No	No	1 < 2	No
Lymphocytes	Increase*	No	Indicine > Taurine*	No	No
Neutrophils	Increase	Increase*	Indicine < Taurine*	1 < 2	1 > 2
Eosinophils	No	No	No	No	No
Monocytes	Decrease*	No	No	No	No
Basophils	No	No	No	1 < 2*	No
Red Blood Cells	No	Decrease	No	1 < 2	1 < 2
Platelets	Increase	No	No	No	No

[1] Significant difference ($P < 0.05$) or differences * approaching significance ($P < 0.10$) between percentage change in leukocyte cell type between KV treatment and control treatment for Indicine and Taurine, significant treatment effects are noted by the direction of percentage change in the KV treatment for each breed respective to the control treatment.
[2] Significant difference ($P < 0.05$) in percentage change between Indicine and Taurine cattle at any time point. Cell types with significant differences are noted with direction of difference between breeds.
[3] V1D versus V2D is a significant difference ($P < 0.05$) in the percentage change between the vaccination first dose (V1D; days 0-14) and the vaccination second dose (V2D; days 21-35) responses within a breed. Significant differences between V1D and V2D responses are noted with the direction of difference between the two time points.

Table 4.5. MIX Treatment Effects. Effects of MIX treatment on percentage change in blood leukocyte cell profiles in Indicine (n = 3) and Taurine (n = 3) calves at vaccination first dose (V1D; day 0-14) and vaccination second dose (V2D; day 21-35) responses.

Phenotype	Treatment Effect[1]		Breed Difference[2]	V1D vs V2D[3]	
	Indicine	Taurine	Significant Effect	Indicine	Taurine
White Blood Cells	Increase	No	Indicine < Taurine*	1 < 2	No
Lymphocytes	No	No	No	1 < 2	No
Neutrophils	No	Increase	Indicine < Taurine	1 > 2	1 < 2
Eosinophils	No	No	No	No	No
Monocytes	No	Decrease	No	No	No
Basophils	Increase*	Decrease	No	No	No
Red Blood Cells	No	Increase*	Indicine > Taurine	No	No
Platelets	No	Increase*/ Decrease	No	No	No

[1] Significant difference ($P < 0.05$) or * approaching significance ($P < 0.10$) between percentage change in leukocyte cell type between MIX treatment and control treatment for Indicine and Taurine, significant treatment effects are noted by the direction of percentage change in the MIX treatment for each breed respective to the control treatment.
[2] Significant difference ($P < 0.05$) in percentage change between Indicine and Taurine at any time point. Cell types with significant differences are noted with direction of difference between breeds.
[3] V1D versus V2D is a significant difference ($P < 0.05$) in the percentage change between the vaccination first dose (V1D; days 0-14) and the vaccination second dose (V2D; days 21-35) responses within a breed. Significant differences between V1D and V2D responses are noted with the direction of difference between the two time points.

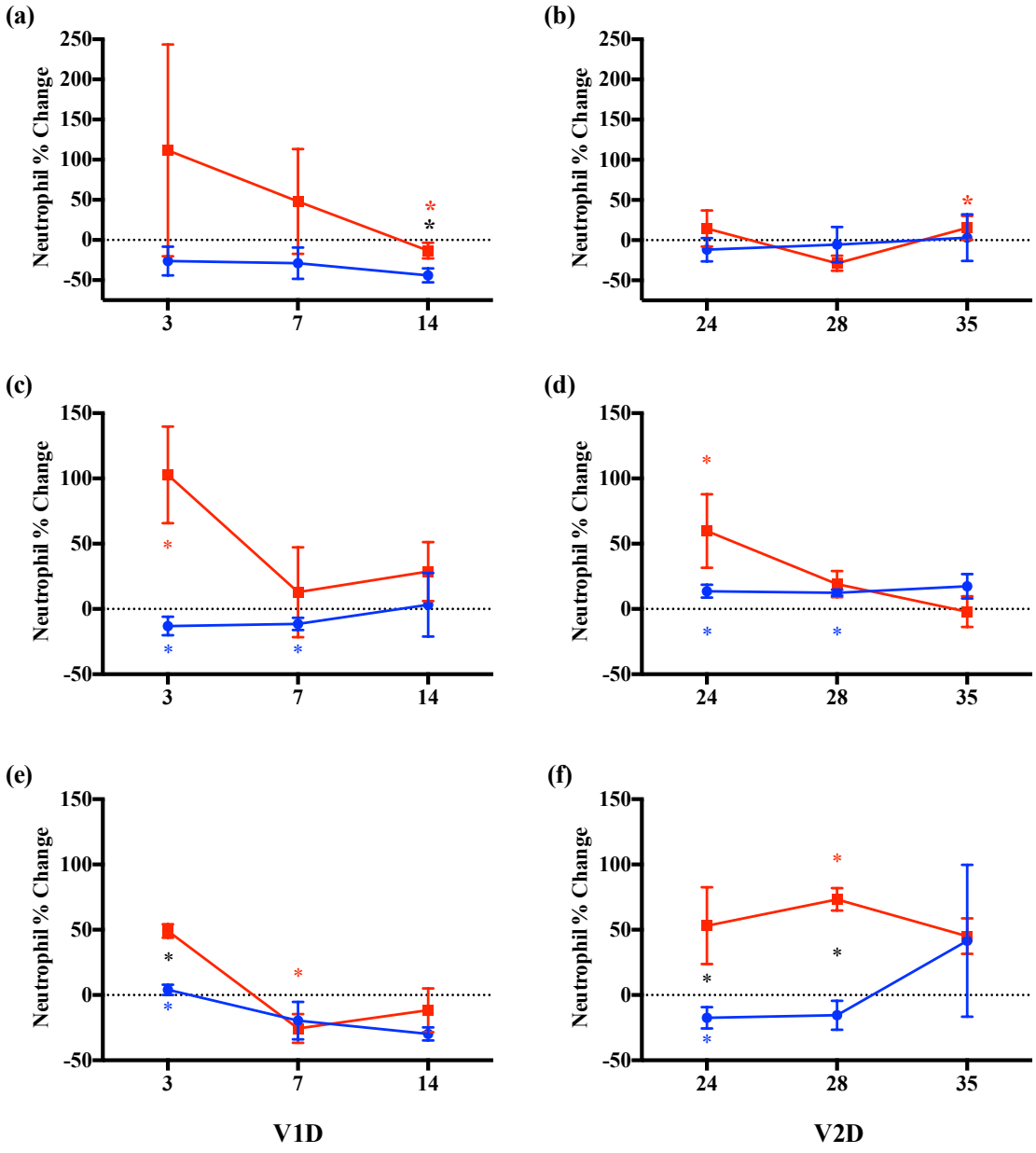


Figure 4.5. Neutrophil Response to Vaccination Treatment. Neutrophil percent change for vaccination first dose (V1D; days 0-14) and vaccination second dose (V2D; days 21-35) responses for MLV, killed vaccine (KV), and MIX treatments: (a) MLV V1D; (b) MLV V2D; (c) KV V1D; (d) KV V1D; (e) MIX V1D; (f) MIX V2D. Percentage change is calculated from day 0 for the V1D response and from day 21 in the V2D response. Data are displayed as mean percentage change with SEM for Indicine (blue, n = 3) and Taurine (red, n = 3). Black * = significant ($P < 0.05$) breed difference; Red/Blue * = Difference between V1D and V2D responses within a breed.

Vaccine Treatment and Dose Affects

The adjuvant (ADJ) did not affect the leukocyte profiles (Table 4.2). The treatment and vaccine dose affected the neutrophil change in the 3 vaccine treatments (Tables 4.3, 4.4, and 4.5). The MLV treatment decreased neutrophils by 40% in indicine cattle at 14 days following the V1D, compared to 7% decrease in taurine cattle at 14 days V2D. The taurine cattle had a 15% higher neutrophil change at 14 days following V2D compared to the V1D change in the MLV treatment (Figure 4.5a and 4.5b). In contrast, the KV increased the neutrophil change at day 28 to the V2D by 22% in indicine cattle and 26% in taurine cattle (Table 4.4). In the MIX treatment the decrease in neutrophils from the MLV vaccine was mitigated (Table 4.5). In the taurine cattle, the neutrophil changes were 3-fold greater than the V2D MLV responses at days 28 and 35 following the booster vaccination (Table 4.5).

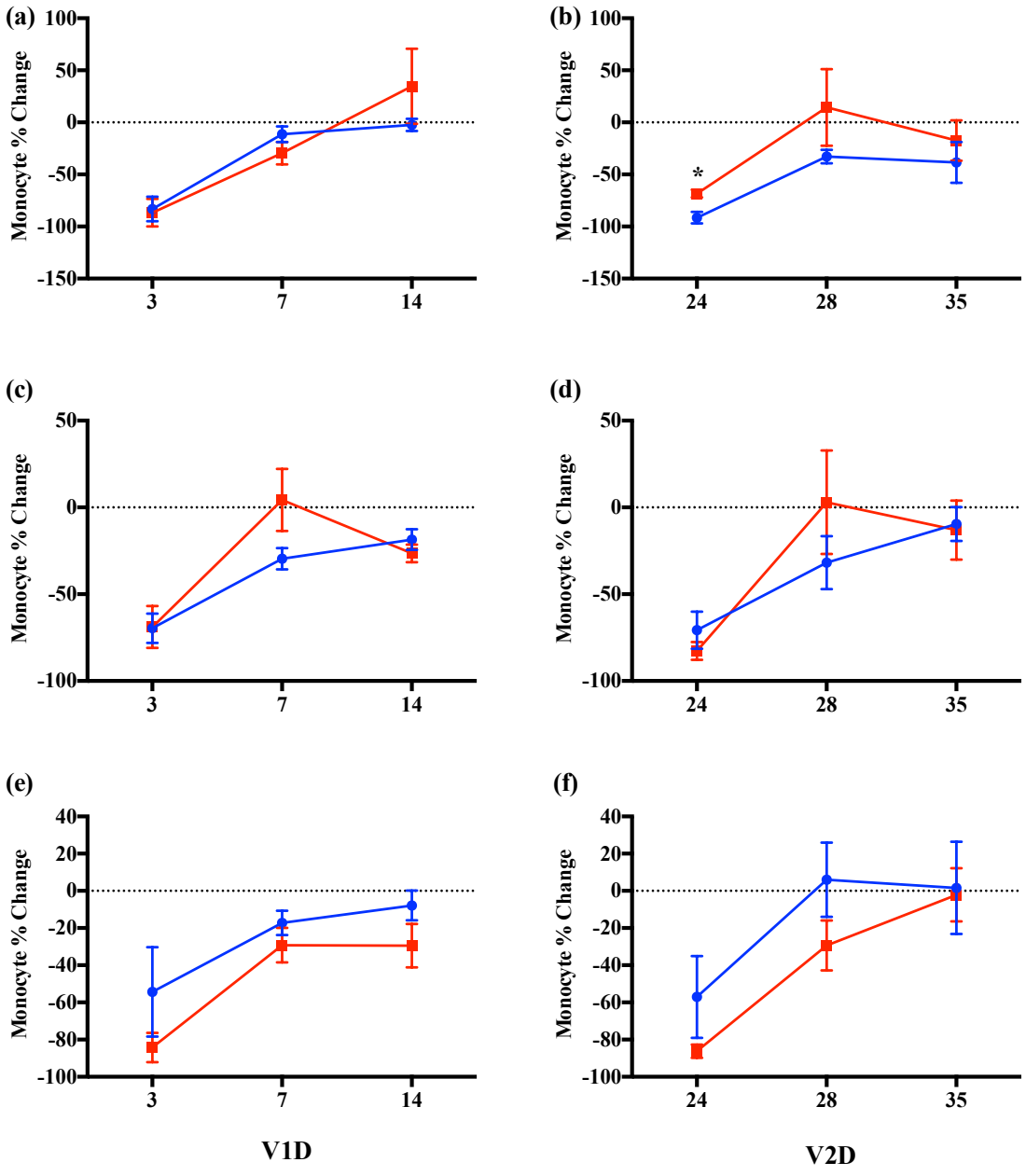


Figure 4.6. Monocyte Response to Vaccination Treatment. Percent change in Monocytes for vaccination first dose (V1D; days 0-14) and vaccination second dose (V2D; days 21-35) responses for MLV, killed vaccine (KV), and MIX treatments: (a) MLV V1D; (b) MLV V2D; (c) KV V1D; (d) KV V1D; (e) MIX V1D; (f) MIX V2D. Percentage change is calculated from day 0 for the V1D response and from day 21 in the V2D response. Data are displayed as mean percentage change with SEM for Indicine (blue, n = 3) and Taurine (red, n = 3). Black * = significant ($P < 0.05$) breed difference; Red/Blue * = Difference between V1D and V2D responses within a breed.

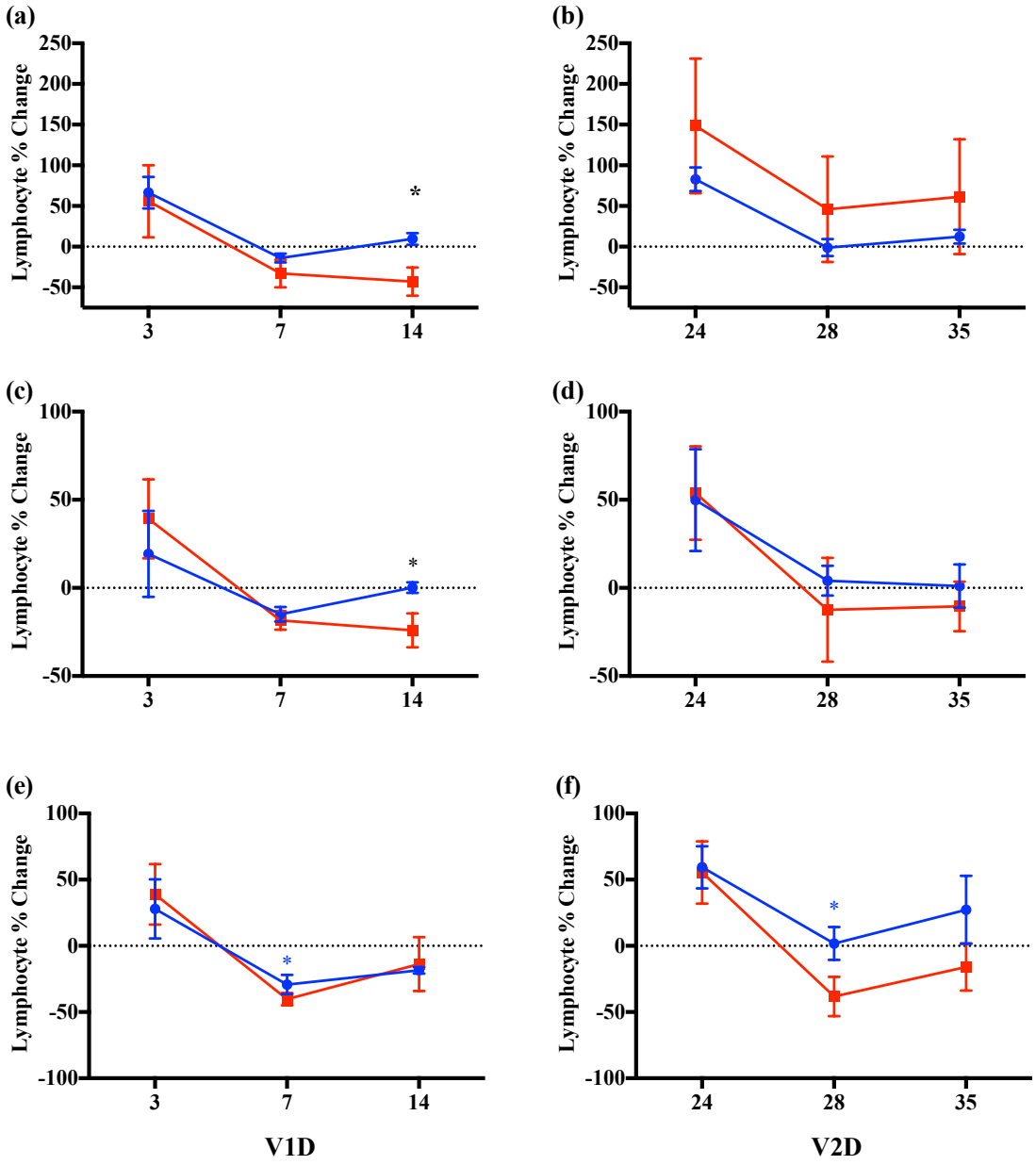


Figure 4.7. Lymphocyte Response to Vaccination Treatment. Percent change in Lymphocytes for vaccination first dose (V1D; days 0-14) and vaccination second dose (V2D; days 21-35) responses for MLV, killed vaccine (KV), and MIX treatments: (a) MLV V1D; (b) MLV V2D; (c) KV V1D; (d) KV V1D; (e) MIX V1D; (f) MIX V2D. Percentage change is calculated from day 0 for the V1D response and from day 21 in the V2D response. Data are displayed as mean percentage change with SEM for Indicine (blue, n = 3) and Taurine (red, n = 3). Black * = significant ($P < 0.05$) breed difference; Red/Blue * = Difference between V1D and V2D responses within a breed.

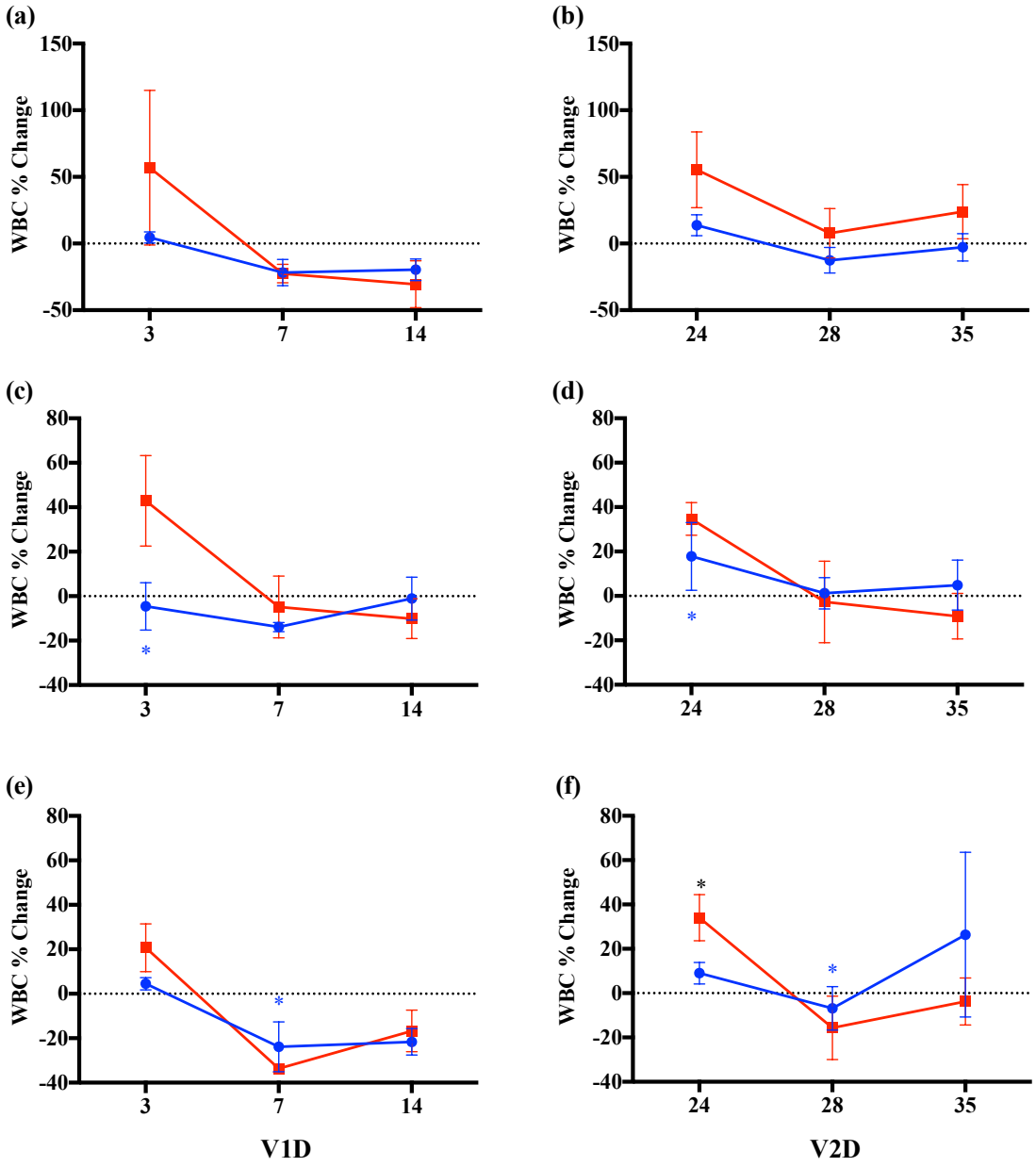


Figure 4.8. WBC Response to Vaccination Treatment. Percent change in White blood cells (WBCs) for vaccination first dose (V1D; days 0-14) and vaccination second dose (V2D; days 21-35) responses for MLV, killed vaccine (KV), and MIX treatments: (a) MLV V1D; (b) MLV V2D; (c) KV V1D; (d) KV V1D; (e) MIX V1D; (f) MIX V2D. Percentage change is calculated from day 0 for the V1D response and from day 21 in the V2D response. Data are displayed as mean percentage change with SEM for Indicine (blue, n = 3) and Taurine (red, n = 3). Black * = significant ($P < 0.05$) breed difference; Red/Blue * = Difference between V1D and V2D responses within a breed.

The V2D KV elicited approximately 20% higher response levels of lymphocytes, neutrophils, and basophils in the indicine calves (Figures 4.7c, 4.7d, 4.5c, 4.5d, 4.9c, and 4.9d) compared to the taurine calves, which had 50% higher changes in neutrophils only to the V1D KV at day 3 (Figures 4.5b and 4.5c). The MIX treatment neutrophil change was reversed compared to that in the KV treatment. The indicine cattle had 20% higher neutrophil response at 3 days V1D in the MIX treatment compared to the V2D response, but had a 60% increase in neutrophils at day 35 V2D (Figures 4.5e and 4.5f). Taurine cattle had 50% less change in neutrophils at day 3 to the V1D than the V2D and an increase of 60 to 70% at days 28 and 35 in the V2D response. Indicine cattle had changes in WBC and lymphocytes that were greater by 18% and 30%, respectively, in the V2D compared to V1D. Eosinophil response was decreased in taurine calves following the MLV vaccination, but the individual variation was too large to detect breed differences (Figure 4.10). RBC change was measured over the course of the study, however changes in RBC were small and more likely due to dehydration than a reflection in adverse response to vaccinations (Figure 4.11).

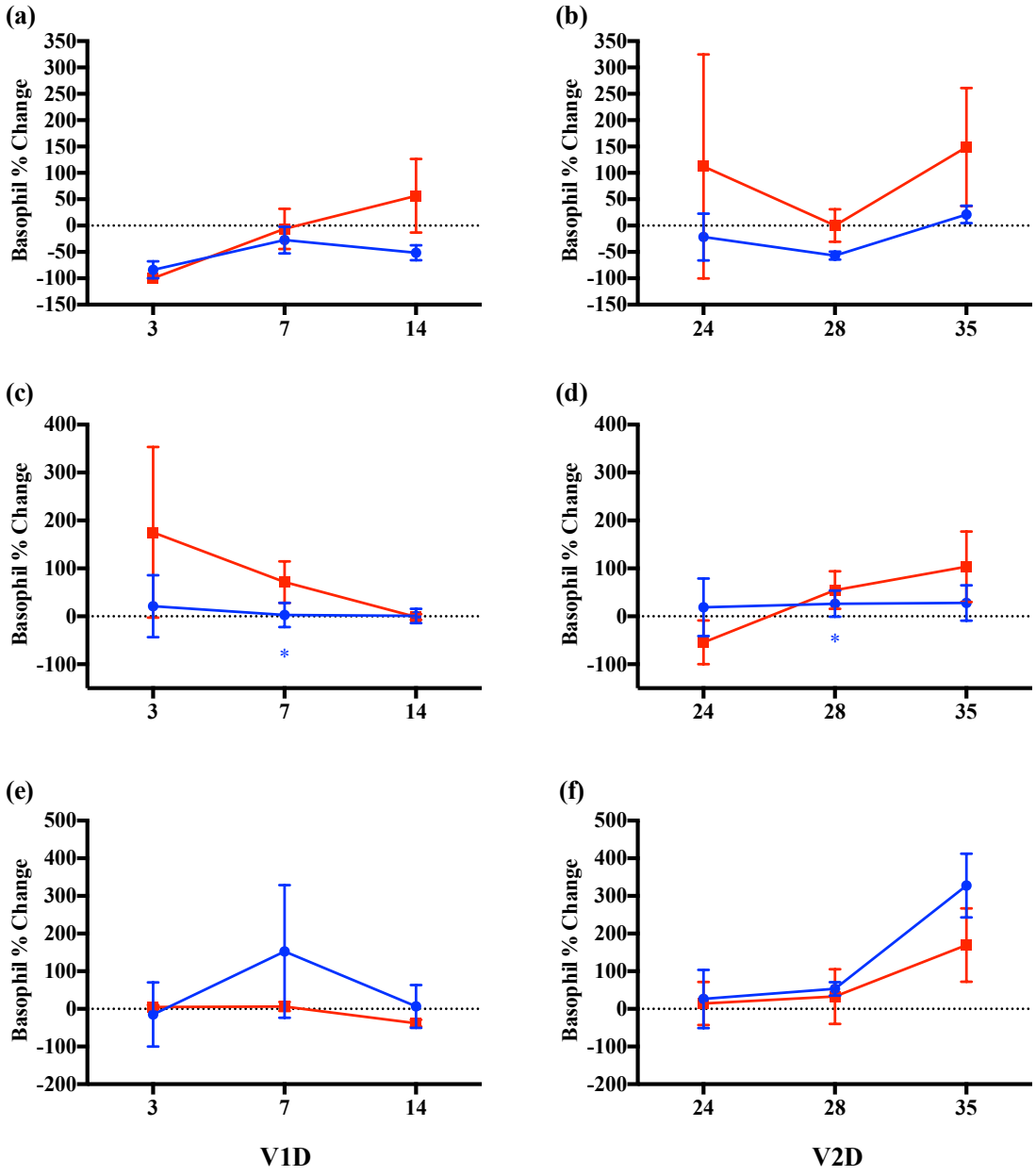


Figure 4.9. Basophil Response to Vaccination Treatment. Basophil percent change for vaccination first dose (V1D; days 0-14) and vaccination second dose (V2D; days 21-35) responses for MLV, killed vaccine (KV), and MIX treatments: (a) MLV V1D; (b) MLV V2D; (c) KV V1D; (d) KV V1D; (e) MIX V1D; (f) MIX V2D. Percentage change is calculated from day 0 for the V1D response and from day 21 in the V2D response. Data are displayed as mean percentage change with SEM for Indicine (blue, n = 3) and Taurine (red, n = 3). Black * = significant ($P < 0.05$) breed difference; Red/Blue * = Difference between V1D and V2D responses within a breed.

Humoral and Cell-mediate Immune Response

Antibody titers for BVDV type 2 exceeded type 1a titers for all treatments. The BVDV type 1a response was detected by day 14 in Charolais calves for KV and MIX treatments (Table 4.6). More Charolais calves had detectable titers to BVDV type 2 than the Brahman calves (Table 4.6). Both breeds had increased numbers of animals with detectable BVDV type 2-specific antibody titers than BVDV type 1a-specific antibody titers in all treatments (Table 4.6). The MLV treatment elicited the greatest number of animals with detectable BVDV type 2-specific antibody titers regardless of breed, while the MIX treatment elicited the largest number of responders for the BVDV 1a-specific antibody titers (Table 4.6).

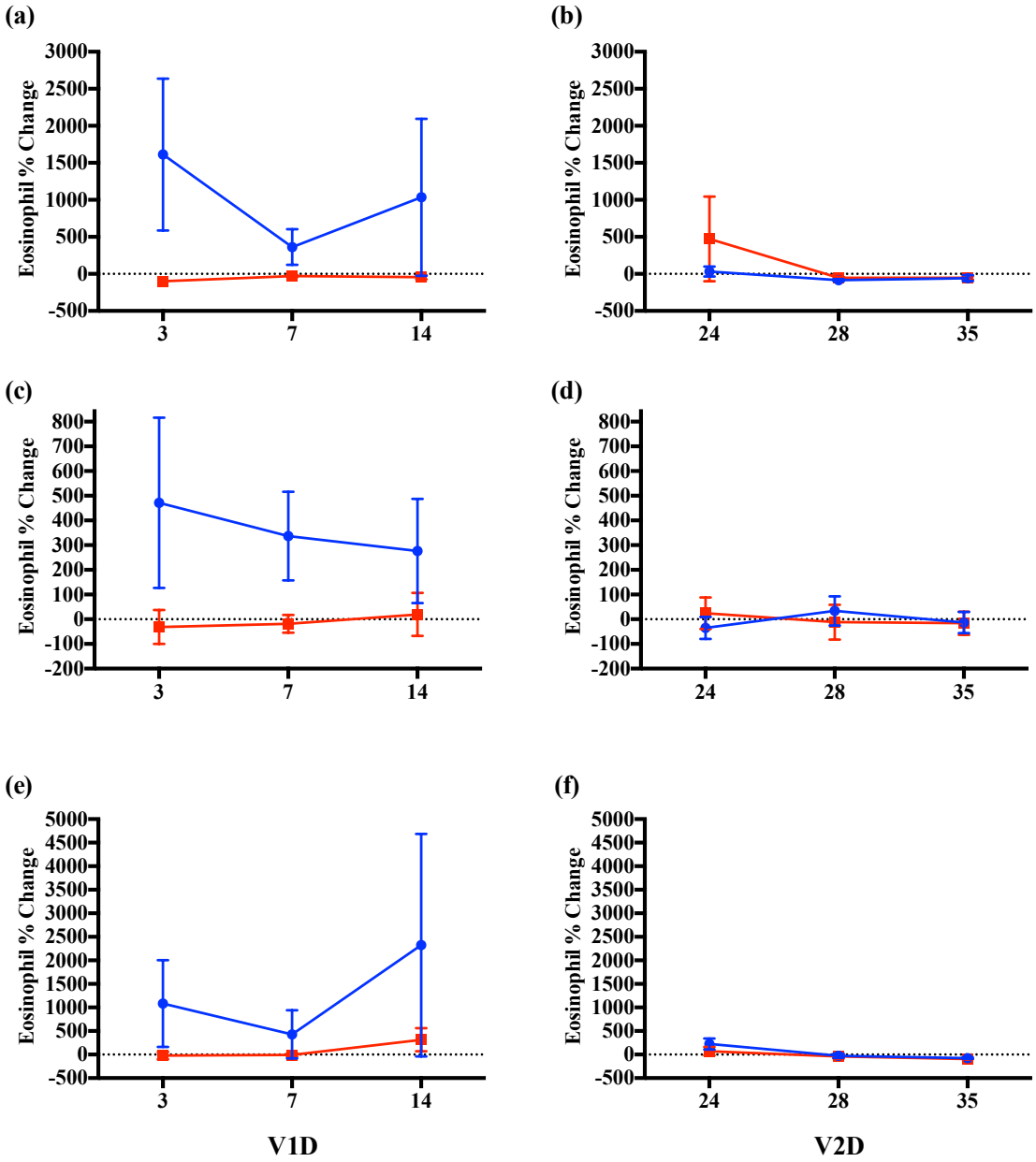


Figure 4.10. Eosinophil Response to Vaccination Treatment. Eosinophil percent change for vaccination first dose (V1D; days 0-14) and vaccination second dose (V2D; days 21-35) responses for MLV, killed vaccine (KV), and MIX treatments: (a) MLV V1D; (b) MLV V2D; (c) KV V1D; (d) KV V1D; (e) MIX V1D; (f) MIX V2D. Percentage change is calculated from day 0 for the V1D response and from day 21 in the V2D response. Data are displayed as mean percentage change with SEM for Indicine (blue, n = 3) and Taurine (red, n = 3). Black * = significant ($P < 0.05$) breed difference; Red/Blue * = Difference between V1D and V2D responses within a breed.

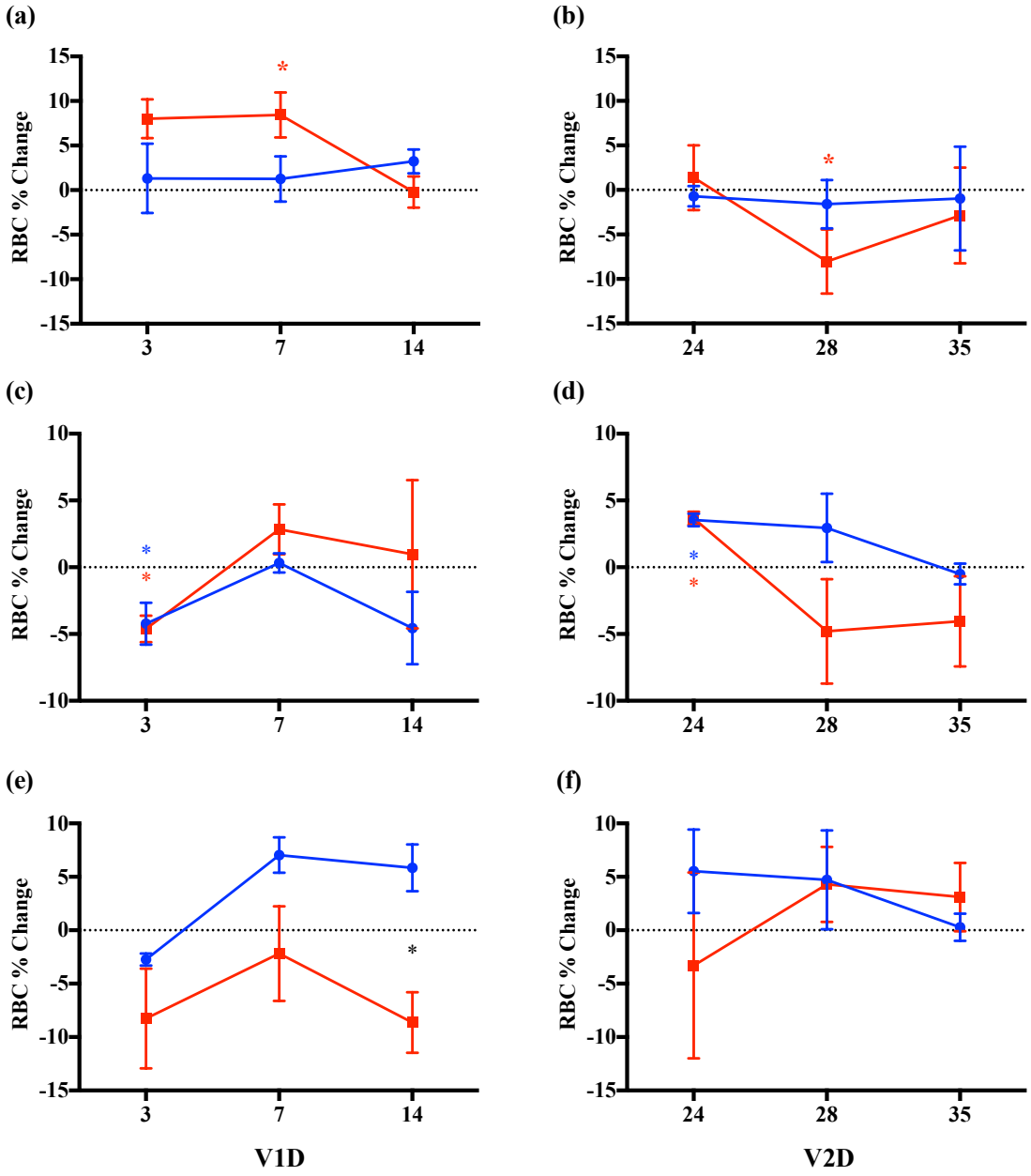


Figure 4.11. RBC Response to Vaccination Treatment. Red blood cell (RBC) percent change for vaccination first dose (V1D; days 0-14) and vaccination second dose (V2D; days 21-35) responses for MLV, killed vaccine (KV), and MIX treatments: (a) MLV V1D; (b) MLV V2D; (c) KV V1D; (d) KV V1D; (e) MIX V1D; (f) MIX V2D. Percentage change is calculated from day 0 for the V1D response and from day 21 in the V2D response. Data are displayed as mean percentage change with SEM for Indicine (blue, n = 3) and Taurine (red, n = 3). Black * = significant ($P < 0.05$) breed difference; Red/Blue * = Difference between V1D and V2D responses within a breed.

Table 4.6. Breed Variation in Antibody Titer. Counts of animals with detectable serum neutralization titers for modified live virus (MLV) vaccine, killed vaccine (KV), and the MIX treatments within breed for BVDV types 1a and 2 at days 14, 21, and 42 post first vaccination.

		Indicine			Taurine		
Treatment		**14**	**21**	**42**	**14**	**21**	**42**
BVDV 1a	**MLV**	1/3	1/3	1/3	1/3	0/3	0/3
	KV	1/3	1/3	1/3	3/3	0/3	0/3
	MIX	0/3	1/3	2/3	3/3	2/3	1/3
BVDV 2	**MLV**	2/3	3/3	3/3	3/3	3/3	3/3
	KV	2/3	2/3	1/3	3/3	1/3	1/3
	MIX	2/3	2/3	3/3	2/3	2/3	3/3

The baseline BVDV response was compared between the breeds at day 0, prior to vaccination (Figure 4.12). Taurine calves had significantly more IFN-γ-secreting T-cells prior to vaccination than indicine calves and more individual variation was observed within the taurine calves (Figure 4.12a). No differences were observed between breeds in the number of BVDV specific proliferating T-cells (Figure 4.12b). The BVDV-specific T-cell response measured IFN-γ-secreting T-cells and no differences were observed between breeds or treatments due to the large variation among samples (Figure 4.13). T-cell response was also measured by T-cell proliferation, similar to the EliSpot assay, but again variation among individual samples was too large to identify significant differences between breeds or among treatments (Figure 4.14).

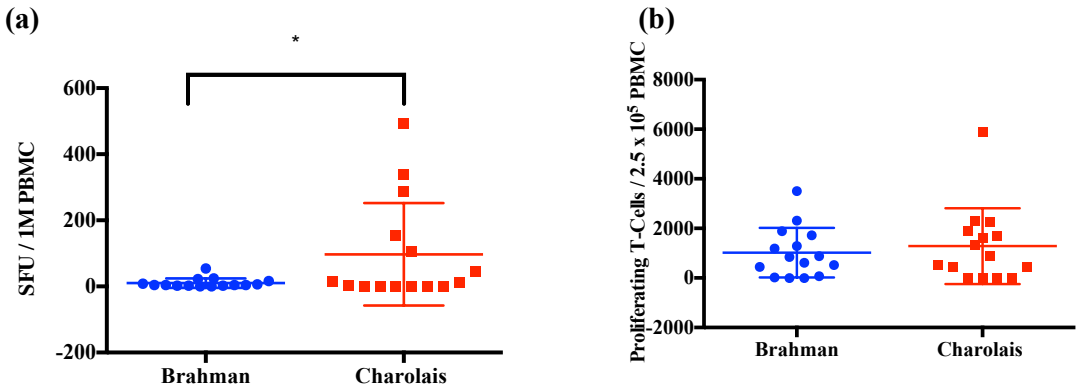


Figure 4.12. Baseline Cell-Mediated Breed Responses. Differences in T-cell responses for **(a)** number of IFN-γ-secreting cells per 1 million PBMCs and **(b)** number of proliferating T-cells when stimulated by BVDV peptides per 2.5 x 10^5 PBMCs on day 0, prior to vaccination, in Brahman (Blue) versus Charolais (Red). Data are displayed as means + SEM (n = 15) for each breed. * = Significant ($P < 0.05$) breed difference.

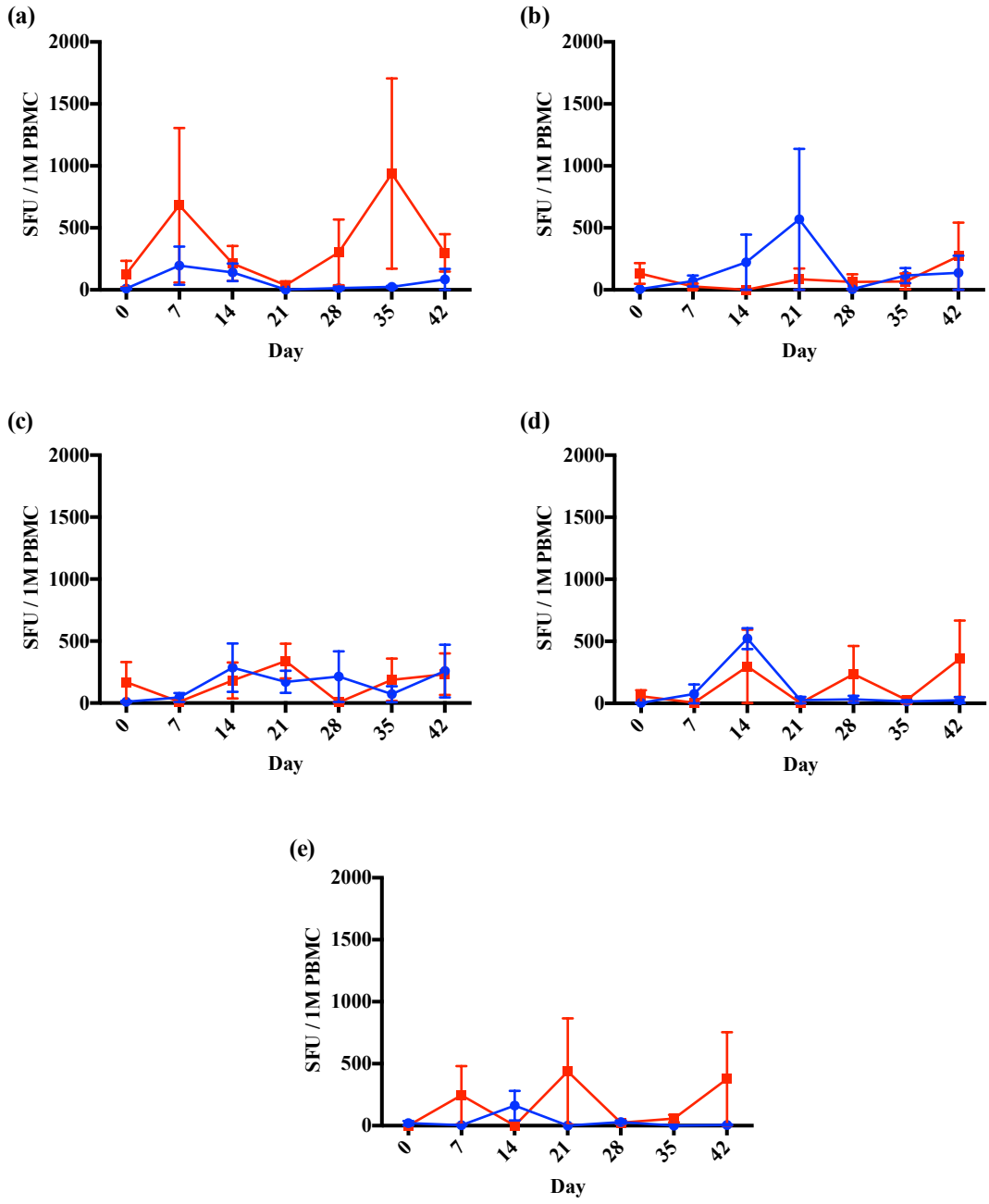


Figure 4.13. IFN-γ-Secreting T-cells Response to Vaccination. Cellular immune response following vaccination in Brahman and Charolais calves from days 0 to 42, measured weekly. Calves were immunized on days 0 and boosted on day 21. The number of IFN-γ-secreting cells per 1 million PBMCs in Brahman (blue) versus Charolais (red) displayed by treatment: **(a)** Control, **(b)** adjuvant, **(c)** MLV, **(d)** killed vaccine, and **(e)** MIX. Data are displayed with means + SEM for 3 calves per Breed-treatment group.

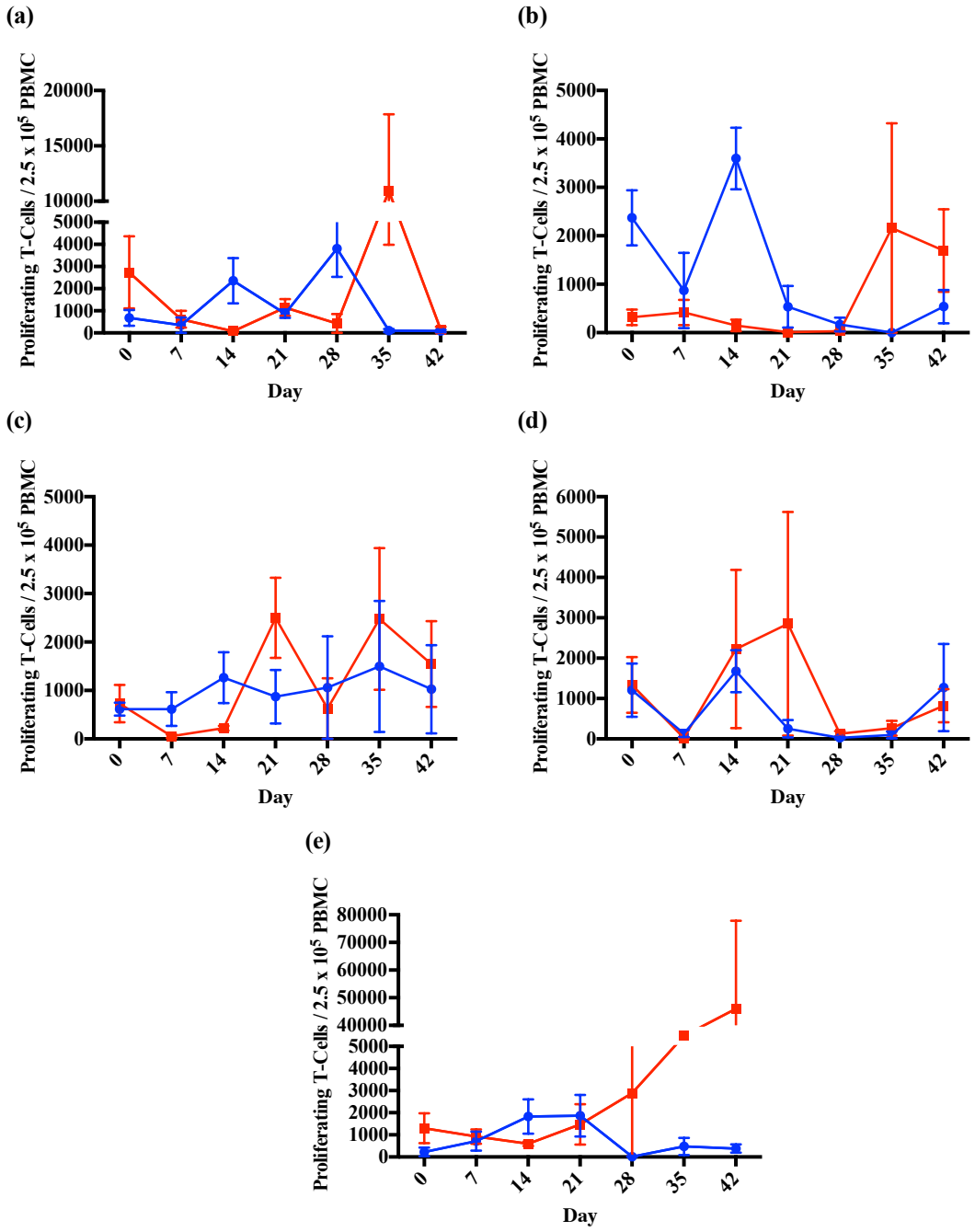


Figure 4.14. T-cell Proliferation in Response to Vaccination. Cellular immune response following vaccination in Brahman and Charolais calves from days 0 to 42, measured weekly. Calves were immunized on days 0 and boosted on day 21. The number of proliferating T-cells when stimulated by BVDV peptides per 2.5 x 10^5 million PBMCs in Brahman (blue) versus Charolais (red) by treatment: **(a)** Control, **(b)** adjuvant, **(c)** MLV, **(d)** killed vaccine, and **(e)** MIX. Data are displayed with means + SEM (n=3) calves per Breed-treatment group.

Discussion

Differences were present in the leukocyte profiles prior to vaccination, which may affect the responses to vaccination that can be achieved. Neutrophil changes were different between the two sub-species hosts, with taurine cattle having the largest change in circulating neutrophils. The animal-to-animal variation was too large to identify differences in cell-mediated responses between breeds, but indicated that variation among individuals affects immune responses to vaccination. Not only was variation in immune response observed, but also significant breed variations were detected in circulating leukocyte profiles that may be important in determining the response to vaccination or infection.

Leukocyte profiles differed between the two sub-species, similar to those reported (MACEDO *et al.* 2013), and lymphocyte percentages were similar to those previously reported between the two breeds (MACEDO *et al.* 2013). However, lymphocytes increased in the indicine calves in response to vaccination while no change was observed in the taurine calves after vaccination. Baseline neutrophil differences were significantly different from those previously reported (MACEDO *et al.* 2013); the Brahman calves had approximately 2-fold increase in neutrophils compared to those reported in other indicine breeds (MACEDO *et al.* 2013). Differences in leukocyte profiles between indicine and taurine breeds may influence the immune response to vaccinations, and may be an important consideration for improved vaccine efficacy.

In indicine cattle, we observed a generalized, non-specific and transitory drop in platelets, regardless of treatment or exposure. The biological significance of this unique response is unknown, although it has been documented that platelets can be carriers of BVDV but do not act as a replication reservoir (CORAPI *et al.* 1989). Taurine platelet levels were similar to levels reported in the literature (KNOWLES *et al.* 2000; RIDPATH 2003). Platelet levels significantly changed from day 0 at vaccination to 3 days post-vaccination in indicine cattle but remained constant in the taurine cattle. Platelets have historically been associated with homeostasis, however due to the early appearance of platelets at sites of injury and infection, it has been proposed that platelets may play an important role in innate immune response (ASSINGER 2014; HERTER *et al.* 2014; ROSSAINT AND ZARBOCK 2015; YADAV AND KOR 2015). Platelets release chemokines and cytokines, specifically P-selectin and CD40L, which are important for recruitment of neutrophils, antigen presenting cells, and lymphocytes (HERTER *et al.* 2014; YADAV AND KOR 2015) and may play a role in recruiting lymphocytes and neutrophils in the innate immune response. The variation we observed in platelet response may be partially responsible for the increased numbers of lymphocytes observed in indicine calves.

Neutrophils are the most common type of WBC circulating in the blood stream, and the most abundant WBC type observed in indicine calves. Neutrophils are first responder cells that release cytokines and chemokines to alert and recruit antigen presenting cells to sites of infection (SRIKUMARAN *et al.* 2007; LYNCH *et al.* 2010; ISOBE *et al.* 2012; HAMPTON *et al.* 2015; LIM *et al.* 2015). The more abundant neutrophil cell population in

indicine calves may promote more efficient antigen presentation of the virus from the vaccination. Neutrophil recruitment and depletion have been associated with respiratory disease in other species, and the abundance of neutrophils may contribute to disease resistance in indicine cattle (MARUFU *et al.* 2014; MCQUEEN *et al.* 2014). The Brahman's ability to maintain higher cell populations might play a role in eliciting a faster and more robust innate immune response, which allows for quick detection and potentially reduced risk of disease (SRIKUMARAN *et al.* 2007; MACEDO *et al.* 2013). Little is known about how neutrophils recruit T-cells, but it was recently shown that neutrophils are important for T-cell recruitment (HAMPTON *et al.* 2015; LIM *et al.* 2015).

Adjuvants are used to enhance immune response to killed vaccines (RIDPATH *et al.* 2010) and we used an adjuvant control without the viral pathogens. No treatment response to the adjuvant was observed, nor did we observe any leukocyte differences in breed response to the adjuvant except in platelets. These results indicate that the viral antigens, and not a response to the adjuvant, drove any response observed from the KV treatment.

Cortisol is often used as a stress indicator and cortisol levels are known to play a role in acute phase immune response and inflammation (CARROLL AND BURDICK SANCHEZ 2013; HUGHES *et al.* 2014; CARROLL *et al.* 2015). Elevated cortisol concentrations in indicine cattle did not appear to increase immunosuppression of WBCs associated with vaccine-induced immunosuppression. If cortisol concentrations were

immunosuppressive in indicine calves, we would have expected to see suppression equally across the three treatments, not limited to just the MLV treatment.

Decreases in neutrophils, basophils, monocytes, and eosinophils in response to the MLV treatment suggest that the MLV treatment has immunosuppressive effects on innate immunity. The decrease in taurine cattle neutrophils was 3-fold less than that observed in the indicine calves, suggesting that the taurine cattle might be better able to mitigate the immunosuppression than indicine cattle. The immunosuppression in indicine calves might also be confounded with the increased serum cortisol concentrations but these effects could not be separately analyzed in this study. Similar immunosuppression effects from MLV vaccines have been previously reported (LIANG *et al.* 2006; KELLING *et al.* 2007; FULTON 2015) but not compared between subspecies. The observed responses to the MIX treatment suggest that an initial vaccination with a KV product may help mitigate some immunosuppression effects from the MLV vaccine. The large amount of individual variation observed precluded the analysis of the T-cell response and therefore it was not possible to compare immunosuppression effects on adaptive immune response.

RBCs do not have a known immunological function for response to vaccination. Changes that were observed in RBCs, while significant, were small in comparison to the changes observed in blood leukocytes. RBC changes may be an indication of dehydration in animals at various time points in the study versus a response to the

vaccination. While there is no evidence for the role of RBCs in response to vaccination, they serve as indicators for BRD at-risk animals (RICHESON *et al.* 2013b).

Our results did not indicate a difference in T-cell response between indicine and taurine breeds. To date, a T-cell measurement has not been identified that can correlate vaccine induced T-cell response with protection derived from the vaccination (CORTESE *et al.* 1998; FURMAN AND DAVIS 2015). The viral strains present in the vaccines used may not have been virulent enough or administered at high enough doses to elicit a T-cell response (COLLEN AND MORRISON 2000; ENDSLEY *et al.* 2003; REBER *et al.* 2006; LIANG *et al.* 2008; PLATT *et al.* 2009; CHASE 2013). In addition, the cell-mediated assays may have been more informative on cell-sorted lymphocytes. The ratios of $CD8^+$, $CD4^+$, and gamma-delta T-cells may differ between the breeds and sub-populations may change differently depending on the vaccine type received. However, these changes were unobservable due to the total lymphocyte population that was used for the experiment. Subspecies PBMC proliferation differences may be more detectable following a challenge, as no breed differences were detected for vaccine response. In the cell-mediated and humoral immune response, a large amount of animal-to-animal variation was detected, as has been reported by other groups (RIDPATH 2005; GLASS *et al.* 2012; HUGHES *et al.* 2014). A portion of the variation may be attributed to T-cell responses to environmental pathogens unrelated to the vaccination (FURMAN AND DAVIS 2015). Animal-to-animal variation has often been ignored due to the challenge and expense of phenotype collection; however, the results observed here and the breed variation

observed by (GLASS *et al.* 2012) suggest that the animal-to-animal variation may be critical to understanding host/pathogen interactions and for improved vaccine design, and therefore warrants further investigation.

In summary, our results indicate that differences in blood leukocyte profiles between the two subspecies may influence the immune response developed to vaccination and pathogen exposures. The indicine cattle had more total leukocytes in the blood, but also had greater reductions in leukocytes in response to the vaccination, especially in the MLV treatment. The neutrophil loss in response to the MLV treatment suggests that indicine cattle were more sensitive to immunosuppression. The MIX treatment mitigated more of the immunosuppression in the indicine cattle than in the taurine cattle, suggesting that if the MIX protocol is as protective as an MLV vaccination protocol, the MIX protocol may reduce some of immunosuppressive effects of the two-shot MLV vaccination strategy. Differences in cell-mediated immunity were not detectable due to the large amounts of individual variation. For improved vaccine efficacy, optimizing vaccine response to overcome individual variation may be critical for improved vaccine efficacy as well as improved techniques for measuring cell-mediated responses. Variations observed in breed differences in responses to vaccination and the immunosuppression effects may indicate that management strategies may need to be different for indicine and taurine breeds to optimize disease prevention and reduce production losses.

CHAPTER V

SUMMARY AND CONCLUSIONS

The MHC is a key determinant for disease resistance and susceptibility (PRICE *et al.* 1999), and is of interest to animal breeders as a target for genetic selection of disease resistance and improved immune response (AMILLS *et al.* 1998). Some population-based GWAS have failed to detect associations of disease phenotypes with variants in BoLA although such associations are often made using pedigreed animal resources. BoLA remains incompletely characterized, raising the possibility that SNPs on commercially available microarrays may not capture all of the diversity present in the MHC as observed in this study and by others (CODNER *et al.* 2012; SCHWARTZ AND HAMMOND 2015).

Results from the characterization of BoLA in this study revealed that more diversity is present in the BoLA region of Holsteins than was predicted from analysis of homozygotes using a 50K SNP chip. The underlying haplotype structure was consistent with the predicted 50K homozygous haplotypes. The no call SNPs found in "homozygous" animals suggests that other genomic variation may further define BoLA haplotypes. The frequency of no calls at any one SNP position varied from rare singlets, observed in only one animal to common no calls observed in many animals. The singlets are of particular interest because of their abundance, but they need to be independently confirmed to exclude experimental error as the source of no call. The BovineHD genotyping data revealed 3 times the number of haplotypes, arranged into 38 haplotype

groups, compared to the 13 haplotypes observed on the 50K SNP chip. Due to the highly heterogeneous nature of the MHC, studying "homozygous" samples is preferable, but selecting animals at 95% homozygosity failed to resolve clean haplotypes, revealing apparently low levels of diversity that may indicate recent divergence from an ancestral haplotype to generate haplotype groups or be due to genotyping errors. Additional studies will be needed to resolve this question.

Variation in host immune response detected as animal-to-animal variation is commonly reported, but addressing this variation at the population level to understand the individual host immune response requires expensive, often inappropriate studies. The second aim was to characterize host immune response through a BVDV challenge study and to search for variation in immune response to vaccination between *Bos taurus indicus* and *Bos taurus taurus.*

Antibody titer is the industry gold standard for testing vaccine efficacy, yet it is uncertain what titer threshold is needed for protection against a given pathogen. Results from the BVDV challenge study suggest that antibody titer may not be a reliable metric for measuring protective immunity against a BVDV 1b challenge. Finding a protective threshold may be dependent on additional immune responses, especially cell mediated immunity that has not been measured. Visual diagnosis is traditionally used to diagnose respiratory disease in the field. Results from our study indicate that subclinical symptoms of lymphopenia and thrombocytopenia revealed by differential cell counts are

more predictive than is elevated rectal temperature and other visual signs such as nasal discharge, coughing, or behavioral changes. Blood cell counts are not measured in the field and are challenging for diagnosis in traditional practice. The use of rectal temperature alone failed to identify a substantial proportion of ill animals, indicating that current metrics used for diagnostics are not adequate to prevent disease. The MLV vaccine treatment was the most effective in preventing disease at all titers, especially lymphopenia. Higher titers were not indicative of greater protection.

Variation in host immune response to commercial vaccinations was observed between *Bos taurus indicus* and *Bos taurus taurus* calves. The results from this project indicate that the innate immune system is important for initial response to vaccination and differed between the subspecies. Indicine calves have higher baseline leukocyte profiles that may be related to their ability to respond to immune challenges more quickly and robustly. Neutrophils had the largest contribution to the increased leukocyte populations, at 2-fold greater neutrophil population in indicine cattle compared to taurine cattle. Taurine cattle had a 50% greater recruitment of neutrophils following both doses of the vaccination. Immunosuppression was observed from the MLV vaccine, and larger immunosuppressive effects were observed in the indicine cattle than taurine cattle. However, when the indicine calves were vaccinated with a killed vaccine and boosted with a MLV vaccine, the immunosuppressive effects of the MLV vaccine were mitigated. Further studies are needed to test whether the same level of protection is achieved from the two vaccine protocols. No differences in cell-mediated immune

response were observed between the two groups of calves. This observation may reflect the immunosuppression induced in the indicine calves, but more studies will be needed to validate the observation that the two groups did not differ for cell-mediated response. Specifically, cell sorting of lymphocytes (flow cytometric analysis) would provide a cleaner cell population for analysis and provide a more informative picture of the differences in host immunity responses relative to immunity against pathogens. In addition, measuring cytokine expressions in defined cell types may also identify pathways that differ in immune response between the two subspecies.

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APPENDIX 1

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300006703	24630009
23	BovineHD4100016071	24632821
23	BovineHD2300006704	24633842
23	BovineHD2300006705	24634849
23	BovineHD2300006706	24635697
23	BovineHD4100016072	24637354
23	BovineHD2300006707	24638064
23	BovineHD2300006708	24639688
23	ARS-BFGL-NGS-70327	24640422
23	BovineHD2300006709	24642182
23	BovineHD2300006710	24642893
23	BovineHD2300006711	24643655
23	BovineHD2300006712	24644705
23	BovineHD2300006713	24645895
23	BovineHD2300006714	24646591
23	BovineHD2300006715	24647834
23	BovineHD2300006716	24648638
23	BovineHD2300006717	24649496
23	BovineHD2300006718	24651047
23	BovineHD2300006720	24654783
23	BovineHD2300006721	24655440
23	BovineHD2300006723	24657821
23	BovineHD2300006724	24658806
23	BovineHD2300006725	24660220
23	BovineHD2300006726	24661105
23	BovineHD4100016073	24661981
23	BovineHD2300006727	24662804
23	BovineHD2300006728	24663609
23	BovineHD2300006729	24664810
23	BovineHD2300006730	24665722
23	ARS-BFGL-NGS-16284	24667121
23	BovineHD2300006732	24668667
23	BovineHD2300006733	24669690
23	BovineHD4100016074	24671650
23	BovineHD2300006734	24673038
23	BovineHD2300006735	24673947

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300006736	24678292
23	BovineHD2300006737	24679678
23	BovineHD2300006738	24681174
23	BovineHD2300006740	24684608
23	BovineHD2300006741	24685618
23	BovineHD2300006742	24691420
23	BovineHD2300006743	24692423
23	BovineHD2300006744	24693344
23	BovineHD2300006745	24695042
23	BovineHD2300006746	24696543
23	BovineHD2300006747	24698245
23	ARS-BFGL-NGS-16080	24699202
23	BovineHD2300006748	24700163
23	BovineHD2300006749	24701295
23	BovineHD2300006750	24702011
23	BovineHD2300006751	24704325
23	BovineHD2300006753	24709821
23	BovineHD2300006754	24712057
23	BovineHD2300006755	24714307
23	BovineHD2300006756	24717057
23	BovineHD2300006757	24719098
23	BovineHD2300006758	24720595
23	BovineHD2300006759	24721536
23	BovineHD2300006761	24725205
23	BovineHD2300006762	24726507
23	ARS-BFGL-NGS-118217	24727613
23	BovineHD2300006763	24731056
23	BovineHD2300006764	24732244
23	BovineHD2300006765	24733772
23	BovineHD2300006766	24735095
23	BovineHD2300006768	24737567
23	BovineHD2300006769	24738878
23	BovineHD2300006770	24740729
23	BovineHD2300006771	24742229
23	BovineHD2300006772	24744378
23	BovineHD2300006773	24752444
23	BovineHD2300006774	24753998
23	BovineHD2300006775	24755350

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300006776	24756932
23	BovineHD2300006777	24758248
23	BovineHD2300006778	24761176
23	BovineHD2300006779	24762740
23	BovineHD2300006780	24763765
23	BovineHD2300006781	24765131
23	BovineHD2300006782	24766984
23	BovineHD2300006783	24768289
23	BovineHD2300006784	24769761
23	BovineHD2300006785	24771290
23	BovineHD2300006786	24773699
23	BovineHD2300006787	24775092
23	BovineHD2300006788	24777898
23	BovineHD2300006789	24781080
23	BovineHD2300006790	24782553
23	BovineHD2300006791	24788503
23	BovineHD2300006792	24789851
23	BovineHD2300006793	24790726
23	BovineHD2300006794	24791564
23	BovineHD2300006795	24794634
23	BovineHD2300006796	24795642
23	BovineHD2300006797	24796756
23	BovineHD2300006798	24799064
23	BovineHD2300006799	24800183
23	BovineHD2300006800	24801233
23	ARS-BFGL-NGS-31623	24803563
23	BovineHD2300006801	24804696
23	BovineHD2300006802	24807136
23	BovineHD2300006803	24808801
23	BovineHD2300006805	24814679
23	BovineHD2300006806	24820046
23	BovineHD2300006807	24821821
23	BovineHD2300006808	24824269
23	BovineHD2300006810	24826678
23	BovineHD2300006811	24827684
23	BovineHD2300006812	24829021
23	BovineHD2300006813	24830023
23	BovineHD2300006814	24830762

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300006815	24831595
23	BovineHD2300006816	24832785
23	BovineHD2300006817	24834115
23	BovineHD2300006818	24835993
23	BovineHD2300006819	24837280
23	BovineHD2300006820	24838732
23	BovineHD2300006821	24839580
23	BovineHD2300006822	24851815
23	BovineHD2300006823	24853573
23	BovineHD2300006824	24855042
23	BovineHD2300006825	24856453
23	BovineHD2300006826	24858203
23	BovineHD2300006827	24861935
23	BovineHD2300006828	24863491
23	BovineHD2300006829	24864848
23	BovineHD2300006830	24867304
23	ARS-BFGL-NGS-17549	24873758
23	BovineHD2300006832	24875159
23	BovineHD2300006834	24878900
23	BovineHD2300006836	24882591
23	BovineHD2300006837	24883720
23	BovineHD2300006838	24885451
23	BovineHD2300006842	24894468
23	BovineHD2300006843	24895470
23	BovineHD2300006844	24897157
23	BovineHD2300006845	24898238
23	BovineHD2300006846	24899379
23	BovineHD2300006847	24905962
23	BovineHD2300006848	24909322
23	BovineHD2300006849	24915043
23	BovineHD2300006850	24917668
23	BovineHD2300006852	24920066
23	BovineHD2300006853	24921775
23	BovineHD2300006854	24923423
23	BovineHD2300006855	24925134
23	BovineHD2300006856	24926303
23	BovineHD2300006857	24928502
23	Hapmap41584-BTA-56031	24939249

Chromosome	SNP Name	UMD 3.1 Position
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23	BovineHD2300006863	24949588
23	BovineHD2300006864	24950266
23	BovineHD2300006865	24951744
23	BovineHD2300006866	24953073
23	BovineHD2300006867	24953991
23	BovineHD2300006868	24954802
23	BovineHD2300006869	24957378
23	BovineHD2300006870	24958143
23	BovineHD2300006871	24959233
23	ARS-BFGL-NGS-96246	24961006
23	BovineHD2300006872	24961958
23	BovineHD2300006873	24965919
23	BovineHD2300006874	24966617
23	BovineHD2300006875	24968160
23	BovineHD2300006876	24968839
23	BovineHD2300006877	24970079
23	BovineHD2300006878	24971907
23	BovineHD2300006879	24972808
23	BovineHD2300006880	24973441
23	BovineHD2300006882	24975768
23	BovineHD2300006883	24980510
23	BovineHD2300006884	24982522
23	BovineHD2300006885	24986300
23	BovineHD2300006887	24988461
23	ARS-BFGL-NGS-44911	24989231
23	BovineHD2300006888	24990037
23	BovineHD2300006890	24991667
23	BovineHD2300006891	24992660
23	BovineHD2300006892	24996699
23	BovineHD2300006893	24998305
23	BovineHD2300006894	24999318
23	BovineHD4100016076	25002374
23	BovineHD2300006895	25003117
23	BovineHD2300006896	25004001
23	BovineHD2300006897	25005097
23	BovineHD2300006898	25006381
23	BovineHD2300006900	25010197

Chromosome	SNP Name	UMD 3.1 Position
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23	BovineHD2300006902	25013490
23	BovineHD2300006903	25015090
23	BovineHD2300006904	25016031
23	BovineHD2300006905	25018708
23	BovineHD4100016077	25019854
23	BovineHD2300006906	25022172
23	BovineHD2300006907	25022917
23	BovineHD2300006908	25023838
23	BovineHD2300006909	25024681
23	BovineHD2300006910	25026030
23	BovineHD2300006911	25027499
23	BovineHD2300006912	25029399
23	BovineHD2300006913	25031476
23	BovineHD2300006914	25032803
23	BovineHD2300006915	25034720
23	BovineHD2300006916	25035810
23	Hapmap28130-BTA-137222	25038202
23	BovineHD2300006917	25039510
23	BovineHD2300006918	25042152
23	BovineHD2300006919	25043430
23	BovineHD4100016078	25044241
23	BovineHD2300006920	25045082
23	BovineHD2300006921	25046288
23	BovineHD2300006922	25048120
23	BovineHD2300006923	25049320
23	BovineHD2300006924	25050387
23	BovineHD2300006925	25053742
23	BovineHD2300006926	25055903
23	BovineHD2300006927	25057162
23	BovineHD2300006928	25058352
23	BovineHD2300006929	25059682
23	BovineHD2300006930	25061435
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23	BovineHD2300006931	25063361
23	BovineHD2300006932	25065432
23	BovineHD2300006933	25067536
23	BovineHD2300006934	25068712

Chromosome	SNP Name	UMD 3.1 Position
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23	BovineHD2300006936	25072931
23	BovineHD2300006937	25073727
23	BovineHD2300006938	25074632
23	BovineHD2300006940	25078318
23	BovineHD4100016079	25080347
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23	BovineHD2300006943	25084989
23	BovineHD2300006944	25086476
23	BovineHD2300006945	25087827
23	BovineHD2300006946	25088780
23	BovineHD2300006947	25089544
23	BovineHD2300006948	25090571
23	BovineHD2300006949	25096460
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23	BovineHD2300006969	25130342
23	BovineHD2300006970	25133409
23	BovineHD2300006971	25134832
23	BovineHD2300006972	25136120

Chromosome	SNP Name	UMD 3.1 Position
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23	BovineHD2300006973	25137471
23	BovineHD2300006974	25143943
23	BovineHD2300006975	25150217
23	BovineHD2300006976	25152367
23	BovineHD2300006977	25153746
23	BovineHD2300006978	25154615
23	BovineHD2300006979	25155638
23	BovineHD2300006980	25156692
23	BovineHD2300006981	25157840
23	BovineHD2300006982	25158615
23	BovineHD2300006983	25159504
23	BovineHD2300006984	25160407
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23	BovineHD2300006993	25168530
23	BovineHD2300006994	25169530
23	BovineHD2300006995	25171173
23	BovineHD2300006996	25171927
23	BovineHD2300006997	25172989
23	BovineHD2300006998	25177690
23	Hapmap44002-BTA-110636	25178791
23	BovineHD2300006999	25179729
23	BovineHD2300007000	25180801
23	BovineHD2300007001	25182719
23	BovineHD2300007002	25184508
23	BovineHD2300007003	25185451
23	BovineHD2300007004	25187635
23	BovineHD2300007005	25188742
23	BovineHD2300007006	25189883
23	Hapmap32120-BTA-155825	25194666
23	BovineHD2300007007	25202537
23	BovineHD2300007008	25203581
23	BovineHD2300007009	25208222

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007010	25209585
23	BovineHD2300007011	25215395
23	BovineHD2300007012	25217535
23	BovineHD2300007013	25218350
23	BovineHD2300007014	25219315
23	BovineHD2300007015	25220421
23	BovineHD2300007016	25221779
23	ARS-BFGL-NGS-117031	25222914
23	BovineHD2300007017	25224449
23	BovineHD2300007018	25226162
23	BovineHD2300007019	25229107
23	BovineHD2300007020	25232601
23	BovineHD2300007021	25236187
23	BovineHD4100016080	25238852
23	BovineHD2300007022	25241197
23	BovineHD2300007024	25244423
23	BovineHD2300007025	25246301
23	BovineHD2300007026	25248392
23	BovineHD2300007027	25252898
23	BovineHD2300007028	25255521
23	BovineHD2300007029	25256604
23	BovineHD2300007030	25263573
23	BovineHD2300007031	25268437
23	BovineHD2300007032	25315725
23	BovineHD2300007034	25323186
23	BovineHD2300007035	25325230
23	BovineHD2300007037	25329895
23	BovineHD2300007040	25335659
23	BovineHD2300007043	25341782
23	BovineHD2300007044	25343174
23	BovineHD2300007048	25349535
23	BovineHD2300007049	25350763
23	BovineHD2300007050	25351819
23	BovineHD2300007051	25353488
23	BovineHD2300007052	25354853
23	BovineHD2300007053	25361041
23	BovineHD2300007057	25417035
23	BovineHD2300007058	25425442

Chromosome	SNP Name	UMD 3.1 Position
23	ARS-BFGL-NGS-54047	25426985
23	BovineHD2300007059	25428298
23	BovineHD2300007060	25429469
23	BovineHD2300007061	25431568
23	BovineHD2300007062	25433133
23	BovineHD2300007063	25434194
23	BovineHD2300007064	25438003
23	BovineHD2300007065	25439352
23	BovineHD2300007066	25440853
23	BovineHD2300007067	25442124
23	BovineHD2300007068	25443589
23	BovineHD2300007069	25445295
23	BovineHD2300007070	25446685
23	BovineHD2300007071	25447954
23	BovineHD2300007072	25449663
23	BovineHD2300007073	25450746
23	BovineHD2300007074	25452180
23	BovineHD2300007076	25465450
23	ARS-BFGL-NGS-12818	25472436
23	BovineHD2300007077	25475120
23	BovineHD2300007078	25475831
23	BovineHD2300007079	25476950
23	BovineHD2300007080	25478637
23	BovineHD2300007081	25479794
23	BovineHD2300007082	25481868
23	Hapmap49222-BTA-55895	25486358
23	BovineHD2300007083	25497178
23	BovineHD2300007085	25499127
23	BovineHD2300007086	25500330
23	BovineHD2300007088	25502202
23	BovineHD2300007089	25504409
23	BovineHD2300007090	25506208
23	ARS-BFGL-NGS-4203	25507676
23	BovineHD2300007091	25508883
23	BovineHD2300007092	25513202
23	BovineHD2300007093	25516596
23	BovineHD2300007095	25521621
23	BovineHD2300007096	25522681

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007098	25529192
23	BovineHD2300007099	25530841
23	BovineHD2300007100	25535263
23	BovineHD2300007101	25537419
23	BovineHD2300007102	25539699
23	BovineHD2300007103	25541686
23	BovineHD2300007104	25544975
23	BovineHD2300007105	25545687
23	BovineHD2300007106	25546632
23	BovineHD2300007107	25549498
23	BovineHD2300007108	25551614
23	ARS-BFGL-NGS-109142	25552635
23	BovineHD2300007109	25554714
23	BovineHD2300007110	25555847
23	BovineHD2300007111	25557554
23	BovineHD2300007112	25558348
23	BovineHD2300007113	25562202
23	BovineHD2300007114	25563969
23	BovineHD2300007115	25565003
23	BovineHD2300007116	25566123
23	BovineHD2300007117	25567290
23	BovineHD2300007118	25568583
23	BovineHD2300007119	25569895
23	BovineHD2300007120	25571002
23	BovineHD2300007121	25571718
23	BovineHD2300007123	25574353
23	BovineHD2300007124	25576881
23	BovineHD2300007126	25578363
23	BovineHD2300007127	25579823
23	BovineHD2300007128	25583529
23	BovineHD2300007129	25587771
23	BovineHD2300007130	25588922
23	BovineHD2300007131	25589707
23	BTA-56042-no-rs	25590646
23	BovineHD2300007132	25591536
23	BovineHD2300007133	25594106
23	BovineHD2300007134	25626467
23	BovineHD2300007135	25632620

Chromosome	SNP Name	UMD 3.1 Position
23	BTA-118887-no-rs	25642674
23	BovineHD2300007136	25669376
23	BovineHD2300007148	25747610
23	BovineHD2300007162	25846520
23	BovineHD4100016081	25856289
23	BovineHD2300007168	25859762
23	BovineHD2300007169	25860822
23	BovineHD2300007171	25863611
23	BovineHD2300007173	25866835
23	BovineHD2300007174	25869447
23	BovineHD2300015298	25875898
23	BovineHD2300007177	25881173
23	BovineHD2300007194	26287961
23	BovineHD2300007196	26297751
23	BovineHD2300007199	26337243
23	BovineHD2300007201	26361697
23	BovineHD2300007202	26369699
23	BovineHD2300007204	26447194
23	BovineHD2300007216	26583740
23	BovineHD2300007219	26663258
23	BovineHD2300007221	26685681
23	BovineHD2300007222	26688149
23	BovineHD2300007223	26689114
23	BovineHD2300007224	26690095
23	BovineHD2300007225	26692183
23	BovineHD2300007227	26711242
23	BovineHD2300007228	26723063
23	BovineHD2300007229	26725494
23	BovineHD2300007230	26727349
23	BovineHD2300007231	26728505
23	BovineHD2300007232	26729844
23	BovineHD2300007233	26732472
23	BovineHD2300007234	26735115
23	BTA-27247-no-rs	26736263
23	BovineHD2300007235	26737241
23	BovineHD2300007236	26740102
23	BovineHD2300007237	26741107
23	BovineHD2300007238	26742222

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007239	26744351
23	BovineHD2300007240	26745815
23	BovineHD2300007241	26748865
23	BovineHD2300007242	26749849
23	BovineHD2300007243	26750784
23	BovineHD2300007244	26753248
23	BovineHD2300007245	26754246
23	BovineHD2300007247	26760034
23	BovineHD2300007249	26762449
23	BovineHD2300007250	26763157
23	BovineHD2300007251	26771426
23	BovineHD2300007252	26774915
23	BovineHD2300007253	26779513
23	BovineHD2300007254	26780394
23	BovineHD2300007255	26784050
23	BovineHD2300007256	26786177
23	BovineHD2300007257	26787873
23	BovineHD2300007258	26789529
23	BovineHD2300007260	26795622
23	BovineHD2300007261	26799991
23	BovineHD2300007262	26803751
23	BovineHD2300007263	26805639
23	BovineHD2300007264	26808474
23	ARS-BFGL-NGS-85480	26812296
23	BovineHD2300007265	26815135
23	BovineHD2300007268	26822064
23	BovineHD2300007269	26826341
23	BovineHD2300007270	26827418
23	BovineHD2300007271	26836195
23	BovineHD2300007272	26837639
23	BovineHD2300007273	26838966
23	BovineHD2300007274	26839964
23	BovineHD2300007275	26841834
23	BovineHD2300007277	26845341
23	BovineHD2300007278	26847193
23	BovineHD2300007279	26850625
23	BovineHD2300007280	26853861
23	BovineHD2300007281	26855288

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007282	26856460
23	BovineHD2300007283	26861195
23	BovineHD2300007284	26866547
23	BovineHD2300007285	26868929
23	BovineHD2300007286	26873233
23	BovineHD2300007287	26874713
23	BovineHD2300007289	26877833
23	BovineHD2300007291	26891901
23	BovineHD2300007292	26892972
23	BovineHD2300007293	26894115
23	BovineHD2300007294	26895106
23	BovineHD2300007295	26897142
23	BovineHD2300007297	26899362
23	BovineHD2300007298	26901293
23	BovineHD2300007299	26902904
23	BovineHD2300007300	26904144
23	BovineHD2300007301	26905056
23	BovineHD2300007303	26907964
23	BovineHD2300007304	26913683
23	BovineHD2300007305	26915139
23	BovineHD2300007308	26918397
23	BovineHD2300007309	26919318
23	BovineHD2300007310	26920473
23	BovineHD2300007311	26921610
23	BovineHD2300007314	26924877
23	BovineHD2300007315	26925552
23	BovineHD2300007316	26926532
23	BovineHD2300007317	26927646
23	BovineHD2300007318	26928623
23	BovineHD2300007319	26929522
23	BovineHD2300007342	26952136
23	BovineHD2300007343	26953748
23	BovineHD2300007344	26954890
23	BovineHD2300007346	26957120
23	BovineHD2300007347	26958140
23	BovineHD2300007348	26959389
23	BovineHD2300007349	26960696
23	BovineHD2300007350	26962122

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007351	26965097
23	BovineHD2300007352	26968410
23	BovineHD2300007353	26969747
23	BovineHD2300007354	26970648
23	BovineHD2300007355	26972152
23	BovineHD2300007356	26973749
23	BovineHD2300007357	26974704
23	BovineHD2300007358	26975583
23	BovineHD2300007359	26976778
23	BovineHD2300007360	26978033
23	BovineHD2300007361	26979851
23	BovineHD2300007362	26981831
23	BovineHD2300007363	26983311
23	BovineHD2300007364	26985409
23	BovineHD2300007366	26989889
23	BovineHD2300007367	26991816
23	BovineHD2300007368	26993631
23	ARS-BFGL-NGS-96349	26995067
23	BovineHD2300007369	26996541
23	BovineHD2300007371	27002117
23	BovineHD2300007372	27003561
23	BovineHD2300007373	27004578
23	BovineHD2300007374	27005424
23	BovineHD2300007375	27006149
23	BovineHD2300007376	27007738
23	BovineHD2300007377	27009252
23	BovineHD2300007378	27010815
23	BovineHD2300007379	27012000
23	BovineHD2300007380	27013524
23	BovineHD2300007381	27014708
23	ARS-BFGL-NGS-24349	27018207
23	BovineHD2300007382	27022099
23	BovineHD2300007383	27026670
23	BovineHD2300007384	27030821
23	BovineHD2300007385	27032818
23	BovineHD2300007386	27034574
23	BovineHD2300007387	27035766
23	BovineHD2300007388	27041670

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007389	27045558
23	BovineHD2300007390	27050988
23	BovineHD2300007391	27055000
23	BovineHD2300007392	27057476
23	ARS-BFGL-BAC-28338	27058749
23	BovineHD2300007393	27059651
23	BovineHD2300007395	27064496
23	BovineHD2300007396	27065334
23	BovineHD2300007397	27066522
23	BovineHD2300007398	27067492
23	BovineHD2300007399	27068748
23	BovineHD2300007400	27071539
23	BovineHD4100016083	27074289
23	BovineHD2300007401	27075222
23	BovineHD2300007403	27085848
23	BovineHD2300007404	27087252
23	ARS-BFGL-NGS-38776	27088825
23	BovineHD2300007405	27091723
23	BovineHD2300007406	27093423
23	BovineHD2300007408	27095495
23	BovineHD2300007409	27096381
23	BovineHD2300007411	27099758
23	BovineHD2300007412	27100953
23	BovineHD2300007413	27101740
23	BovineHD2300007415	27104697
23	BovineHD2300007416	27105520
23	BovineHD2300007417	27108992
23	BovineHD2300007418	27111525
23	BovineHD2300007419	27113231
23	BovineHD2300007421	27117779
23	BovineHD2300007422	27118533
23	BovineHD2300007423	27119259
23	BovineHD2300007424	27119953
23	BovineHD4100016084	27123974
23	BovineHD2300007426	27125428
23	BovineHD2300007427	27129500
23	BovineHD2300007428	27133733
23	BovineHD2300007430	27148192

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007432	27153283
23	BovineHD2300007433	27154412
23	BovineHD2300007434	27155608
23	BovineHD2300007436	27159594
23	BovineHD2300007437	27162050
23	BovineHD2300007438	27164120
23	BovineHD2300007439	27184978
23	BovineHD2300007441	27195210
23	BovineHD2300007442	27197646
23	BovineHD2300007443	27200345
23	BovineHD2300007444	27205595
23	BovineHD2300007445	27207819
23	Hapmap47328-BTA-56087	27215001
23	BovineHD2300007447	27216072
23	BovineHD2300007448	27217994
23	BovineHD2300007449	27219241
23	BovineHD2300007450	27220211
23	BovineHD2300007451	27222004
23	BovineHD2300007452	27223341
23	BovineHD2300007453	27224507
23	BovineHD2300007454	27226548
23	BovineHD2300007455	27227600
23	BovineHD2300007457	27232684
23	BovineHD2300007458	27233847
23	BovineHD2300007459	27235099
23	BovineHD2300007460	27236505
23	BovineHD2300007461	27237731
23	BovineHD2300007462	27238530
23	BovineHD2300007463	27239487
23	BovineHD2300007464	27240576
23	BovineHD2300007465	27241906
23	BovineHD2300007466	27245044
23	BovineHD2300007467	27247752
23	BovineHD2300007468	27252412
23	ARS-BFGL-NGS-113623	27254728
23	BovineHD2300007469	27256554
23	BovineHD2300007470	27257734
23	BovineHD2300007471	27260109

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007472	27264413
23	BovineHD2300007473	27265348
23	BovineHD2300007474	27266507
23	BovineHD2300007475	27270902
23	BovineHD2300007476	27272126
23	BovineHD2300007477	27273920
23	BovineHD2300007478	27277149
23	BovineHD2300007479	27279196
23	BovineHD2300007480	27280328
23	BovineHD2300007481	27282072
23	BovineHD2300007482	27284754
23	BovineHD2300007483	27287932
23	BovineHD2300007484	27292820
23	BovineHD2300007485	27296029
23	BovineHD2300007486	27298470
23	BovineHD2300007487	27303309
23	BovineHD2300007488	27304195
23	BovineHD2300007489	27306151
23	ARS-USMARC-Parent-AY937242-rs17872223	27306795
23	BovineHD2300007490	27307136
23	BovineHD2300007491	27308305
23	BovineHD4100016085	27309436
23	BovineHD2300007492	27310764
23	BovineHD2300007493	27312648
23	BovineHD2300007494	27314277
23	BovineHD2300007495	27315400
23	BovineHD2300007496	27316320
23	BovineHD2300007497	27319081
23	BovineHD2300007498	27320243
23	BovineHD2300007499	27321410
23	BovineHD2300007500	27322716
23	BovineHD2300007501	27323622
23	BovineHD2300007502	27324830
23	BovineHD2300007503	27325996
23	BovineHD2300007504	27328522
23	BovineHD2300007505	27330337
23	BovineHD2300007506	27331719

Chromosome	SNP Name	UMD 3.1 Position
23	ARS-BFGL-NGS-60524	27333007
23	BovineHD2300007507	27334855
23	BovineHD2300007508	27335628
23	BovineHD2300007509	27340413
23	BovineHD2300007510	27343018
23	BovineHD2300007511	27343928
23	BovineHD2300007512	27345117
23	BovineHD2300007513	27347252
23	BovineHD2300007514	27348488
23	BovineHD2300007515	27353887
23	BovineHD2300007516	27356419
23	BovineHD2300007517	27358154
23	BovineHD2300007518	27359646
23	BovineHD2300007519	27361230
23	BovineHD2300007520	27362154
23	BovineHD2300007521	27362992
23	BovineHD2300007522	27364549
23	BovineHD2300007523	27367039
23	BovineHD2300007524	27368531
23	BovineHD2300007525	27371363
23	BovineHD2300007526	27372146
23	BovineHD2300007527	27375669
23	BovineHD2300007528	27377348
23	BovineHD2300007529	27379438
23	BovineHD2300007530	27380362
23	ARS-BFGL-NGS-72442	27383176
23	BovineHD2300007531	27384391
23	BovineHD4100016086	27388628
23	BovineHD2300007532	27389604
23	BovineHD2300007533	27392997
23	BovineHD2300007534	27398872
23	BovineHD2300007535	27399609
23	BovineHD2300007536	27403791
23	BovineHD2300007537	27405801
23	BovineHD2300007538	27407958
23	BovineHD2300007539	27411352
23	BovineHD2300007540	27413323
23	BovineHD2300007541	27414929

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007542	27416286
23	BovineHD2300007543	27417142
23	BovineHD2300007544	27418137
23	BovineHD2300007545	27419614
23	Hapmap57616-rs29026690	27421348
23	BovineHD2300007546	27425803
23	BovineHD2300007547	27426954
23	BovineHD2300007548	27427745
23	BovineHD2300007549	27428525
23	BovineHD2300007550	27429810
23	BovineHD2300007551	27431101
23	BovineHD2300007552	27432460
23	BovineHD2300007553	27433438
23	BovineHD2300007554	27434384
23	BovineHD2300007555	27435444
23	BovineHD2300007556	27436681
23	BovineHD2300007558	27440681
23	BovineHD2300007559	27441874
23	ARS-BFGL-NGS-104658	27444064
23	BovineHD2300007561	27446997
23	BovineHD2300007562	27448196
23	BovineHD2300007563	27449732
23	BovineHD2300007564	27453148
23	BovineHD2300007565	27453893
23	BovineHD2300007566	27455333
23	BovineHD2300007567	27457212
23	BovineHD2300007568	27460294
23	BovineHD2300007569	27461040
23	BovineHD2300007570	27466031
23	BovineHD2300007571	27466779
23	BovineHD2300007572	27468057
23	BovineHD2300007573	27468743
23	BovineHD2300007574	27471312
23	BovineHD2300007575	27473851
23	BovineHD2300007576	27475326
23	BovineHD2300007578	27478366
23	BovineHD2300007579	27479260
23	BovineHD2300007580	27483000

Chromosome	SNP Name	UMD 3.1 Position
23	ARS-BFGL-NGS-2116	27485467
23	BovineHD2300007581	27489373
23	BovineHD2300007582	27491945
23	BovineHD2300007583	27502853
23	BovineHD2300007584	27505572
23	BovineHD2300007585	27507844
23	BovineHD2300007586	27511777
23	BovineHD2300007587	27514690
23	BovineHD2300007588	27515479
23	BovineHD2300007589	27516240
23	BovineHD2300007590	27519462
23	BovineHD2300007591	27521712
23	BovineHD2300007592	27522842
23	BovineHD2300007593	27523519
23	BovineHD2300007594	27524960
23	BovineHD2300007595	27526010
23	BovineHD2300007596	27527199
23	BovineHD2300007597	27528355
23	BovineHD2300007598	27531038
23	BovineHD2300007599	27531933
23	BovineHD2300007600	27533074
23	BovineHD2300007601	27534831
23	BovineHD2300007602	27535678
23	BovineHD2300007603	27537976
23	BovineHD2300007604	27541174
23	BovineHD2300007605	27542680
23	BovineHD2300007606	27543750
23	ARS-BFGL-NGS-105633	27545231
23	BovineHD2300007607	27547345
23	BovineHD2300007608	27548598
23	BovineHD2300007609	27549721
23	BovineHD2300007610	27551472
23	BovineHD2300007611	27552647
23	BovineHD2300007613	27558777
23	BovineHD2300007614	27559688
23	BovineHD2300007615	27561342
23	BovineHD2300007616	27563184
23	BovineHD2300007617	27564388

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007618	27565313
23	BovineHD2300007620	27567835
23	BovineHD2300007621	27569381
23	BovineHD2300007622	27570033
23	BovineHD2300007623	27575149
23	BovineHD4100016088	27575852
23	BovineHD2300007624	27577284
23	BovineHD2300007625	27578386
23	BovineHD2300007626	27581503
23	ARS-BFGL-NGS-43021	27583474
23	BovineHD2300007628	27591615
23	BovineHD2300007629	27592539
23	BovineHD2300007630	27593703
23	BovineHD2300007631	27598352
23	BovineHD2300007632	27600197
23	BovineHD2300007633	27601403
23	BovineHD2300007634	27602122
23	BovineHD2300007635	27612902
23	BovineHD2300007636	27614084
23	BovineHD2300007637	27617926
23	BovineHD2300007638	27627775
23	BovineHD2300007639	27628683
23	BovineHD2300007640	27632381
23	BovineHD2300007642	27634659
23	BovineHD2300007643	27636385
23	BovineHD2300007644	27640249
23	BovineHD2300007645	27641058
23	BovineHD2300007646	27641714
23	BovineHD2300007647	27642362
23	ARS-BFGL-NGS-99242	27644218
23	BovineHD2300007648	27647007
23	BovineHD2300007649	27650286
23	BovineHD2300007650	27652500
23	BovineHD2300007654	27665155
23	BovineHD2300007662	27685577
23	BovineHD2300007668	27700720
23	BovineHD4100016089	27759950
23	BovineHD2300007683	27761424

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007684	27762666
23	BovineHD2300007685	27763861
23	BovineHD2300007686	27765779
23	BovineHD2300007687	27768031
23	BovineHD2300007688	27770884
23	BovineHD2300007689	27772156
23	BovineHD2300007690	27773293
23	BovineHD2300007691	27774225
23	BovineHD2300007692	27775248
23	ARS-BFGL-NGS-11887	27776075
23	BovineHD2300007693	27776739
23	BovineHD4100016090	27777923
23	BovineHD2300007694	27779566
23	BovineHD2300007695	27780571
23	BovineHD2300007696	27781790
23	BovineHD2300007697	27782568
23	BovineHD2300007698	27783309
23	BovineHD2300007699	27784888
23	BovineHD4100016091	27785749
23	BovineHD2300007700	27786582
23	BovineHD2300007701	27787261
23	BovineHD2300007702	27788723
23	BovineHD2300007704	27790745
23	BovineHD2300007705	27791863
23	BovineHD2300007706	27793691
23	BovineHD2300007707	27796803
23	BovineHD2300007708	27798254
23	BovineHD2300007709	27799841
23	BovineHD2300007710	27800971
23	BovineHD2300007711	27801978
23	BovineHD2300007712	27802812
23	ARS-BFGL-NGS-97747	27804037
23	BovineHD2300007713	27804767
23	BovineHD2300007714	27805404
23	BovineHD2300007715	27806531
23	BovineHD2300007717	27809089
23	BovineHD2300007718	27810230
23	BovineHD2300007719	27811394

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007720	27813222
23	BovineHD2300007722	27817320
23	BovineHD2300007723	27818856
23	BovineHD2300007724	27819560
23	BovineHD2300007725	27820535
23	BovineHD2300007726	27821489
23	BovineHD2300007727	27822754
23	BovineHD2300007728	27824286
23	BovineHD2300007729	27825771
23	BovineHD2300007730	27826838
23	BovineHD2300007731	27827644
23	BovineHD2300007732	27829379
23	BovineHD2300007733	27830182
23	BovineHD2300007734	27831407
23	BovineHD2300007735	27834473
23	BovineHD2300007737	27838172
23	BovineHD2300007738	27839366
23	ARS-BFGL-NGS-32979	27841983
23	BovineHD2300007741	27845424
23	BovineHD2300007742	27846802
23	BovineHD2300007743	27849149
23	BovineHD2300007744	27850893
23	BovineHD2300007745	27852114
23	BovineHD2300007746	27853209
23	ARS-BFGL-NGS-1222	27865384
23	BovineHD2300007750	27880661
23	BovineHD2300007751	27882099
23	BovineHD2300007752	27883444
23	BovineHD2300007753	27885887
23	BovineHD2300007754	27891593
23	BovineHD2300007755	27892922
23	BovineHD2300007756	27896008
23	BovineHD2300007757	27897569
23	BovineHD2300007758	27898835
23	BovineHD2300007759	27901813
23	BovineHD2300007760	27907740
23	BovineHD2300007761	27909622
23	Hapmap46836-BTA-55820	27923154

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007763	27927837
23	BovineHD2300007765	27930626
23	BovineHD2300007766	27931339
23	BovineHD2300007767	27933138
23	BovineHD2300007768	27936796
23	BovineHD2300007769	27938049
23	BovineHD2300007770	27938956
23	BovineHD2300007771	27940432
23	BovineHD2300007772	27941262
23	BTA-55821-no-rs	27944066
23	BovineHD2300007773	27944980
23	BovineHD2300007774	27945751
23	BovineHD2300007775	27946647
23	BovineHD4100016092	27947741
23	BovineHD2300007776	27949898
23	BovineHD2300007777	27950777
23	BovineHD2300007778	27951516
23	BovineHD2300007779	27955163
23	BovineHD2300007780	27956201
23	BovineHD4100016093	27957438
23	BovineHD4100016094	27960902
23	BovineHD2300007781	27963661
23	ARS-BFGL-NGS-67719	27965340
23	BovineHD2300007782	27972458
23	BovineHD2300007783	27974568
23	BovineHD2300007784	27975533
23	BovineHD2300007785	27977768
23	BovineHD2300007786	27978526
23	BovineHD4100016095	27979819
23	BovineHD2300007787	27980733
23	BovineHD4100016096	27981857
23	BovineHD2300007788	27983157
23	BovineHD2300007789	27986085
23	BovineHD2300007790	27988741
23	BovineHD2300007791	27990852
23	BTA-55853-no-rs	27992880
23	BovineHD2300007792	27994640
23	BovineHD2300007793	27996706

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007794	27998431
23	BovineHD2300007795	27999936
23	BovineHD2300007796	28001216
23	BovineHD2300007797	28002159
23	BovineHD2300007798	28003213
23	BovineHD2300007799	28004218
23	BovineHD2300007800	28005696
23	BovineHD2300007801	28006677
23	BovineHD2300007802	28009506
23	BovineHD2300007803	28010500
23	BovineHD2300007804	28011554
23	BovineHD4100016097	28013242
23	BovineHD2300007805	28014950
23	BovineHD2300007806	28016282
23	BovineHD2300007807	28017605
23	BovineHD2300007808	28019214
23	ARS-BFGL-NGS-90570	28021047
23	BovineHD2300007810	28023160
23	BovineHD2300007811	28024603
23	BovineHD2300007812	28025573
23	BovineHD4100016098	28026634
23	BovineHD2300007813	28028219
23	BovineHD2300007814	28029991
23	BovineHD2300007815	28030990
23	BovineHD2300007816	28035349
23	BovineHD2300007819	28042557
23	BTA-55867-no-rs	28046330
23	BovineHD2300007821	28050702
23	BovineHD2300007822	28051757
23	BovineHD2300007823	28052917
23	BovineHD2300007824	28054444
23	BovineHD2300007825	28055819
23	BovineHD2300007826	28059521
23	BovineHD2300007827	28060999
23	BovineHD2300007828	28062298
23	BovineHD2300007829	28063685
23	BovineHD2300007830	28065113
23	BovineHD2300007831	28069648

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007832	28070558
23	BovineHD2300007833	28073080
23	BovineHD2300007834	28076030
23	BovineHD2300007835	28078379
23	BovineHD2300007836	28081519
23	BovineHD2300007837	28082954
23	BovineHD2300007838	28085159
23	ARS-BFGL-NGS-4240	28087630
23	BovineHD2300007839	28091160
23	BovineHD2300007840	28092704
23	BovineHD2300007841	28095010
23	BovineHD2300007842	28096736
23	BovineHD2300007843	28100841
23	BovineHD2300007845	28103362
23	BovineHD2300007846	28109619
23	BovineHD2300007847	28113240
23	BovineHD2300007848	28114866
23	BovineHD2300007849	28116117
23	BovineHD2300007850	28118083
23	BovineHD2300007851	28119317
23	BovineHD2300007852	28120329
23	BovineHD2300007853	28121586
23	BovineHD2300007854	28122679
23	BovineHD2300007855	28123643
23	BovineHD2300007856	28125274
23	BovineHD2300007857	28130236
23	BovineHD2300007858	28131399
23	BovineHD2300007859	28132260
23	BovineHD2300007860	28132973
23	BovineHD2300007861	28133833
23	BovineHD2300007862	28136947
23	BovineHD2300007863	28138125
23	BovineHD2300007864	28138976
23	BovineHD2300007865	28140515
23	BovineHD2300007867	28143866
23	BovineHD2300007868	28146155
23	BovineHD2300007869	28147995
23	BovineHD4100016099	28150627

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007870	28154333
23	BovineHD2300007871	28155284
23	BovineHD2300007872	28157824
23	BovineHD2300007873	28162474
23	BovineHD2300007874	28163721
23	BovineHD2300007875	28165674
23	BovineHD2300007876	28171185
23	BovineHD2300007877	28174203
23	BovineHD2300007878	28180181
23	BovineHD2300007879	28181331
23	BovineHD2300007880	28185008
23	BovineHD2300007881	28185806
23	BovineHD4100016100	28188301
23	BovineHD2300007882	28189121
23	BovineHD2300007883	28190859
23	BovineHD2300007884	28192824
23	UA-IFASA-5823	28193501
23	BovineHD2300007885	28195057
23	BovineHD4100016101	28197255
23	BovineHD2300007886	28198327
23	BovineHD2300007887	28200450
23	BovineHD2300007888	28202487
23	BovineHD2300007890	28206185
23	BovineHD2300007891	28207353
23	BovineHD2300007892	28208600
23	BovineHD2300007893	28209499
23	BovineHD2300007894	28210603
23	BovineHD2300007895	28211855
23	BovineHD2300007896	28213347
23	BovineHD2300007897	28215160
23	BovineHD2300007898	28217018
23	BovineHD4100016102	28219172
23	BovineHD2300007900	28221157
23	ARS-BFGL-NGS-113235	28223274
23	BovineHD2300007901	28224143
23	BovineHD2300007902	28226485
23	BovineHD2300007903	28228193
23	BovineHD2300007904	28229677

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007905	28230561
23	BovineHD2300007906	28231327
23	BovineHD2300007907	28236399
23	BovineHD2300007908	28238171
23	BovineHD2300007909	28240434
23	BovineHD2300007910	28242968
23	BovineHD2300007911	28245665
23	BovineHD2300007912	28246767
23	BovineHD2300007913	28248104
23	BovineHD2300007914	28250023
23	BovineHD2300007915	28251058
23	BovineHD2300007916	28253974
23	BovineHD2300007917	28255814
23	BovineHD2300007918	28257816
23	BovineHD2300007919	28258614
23	BovineHD2300007920	28259298
23	BovineHD2300007921	28260420
23	BovineHD2300007922	28261754
23	BovineHD2300007923	28262651
23	BovineHD2300007924	28263639
23	BovineHD2300007925	28264569
23	BovineHD2300007926	28266616
23	BovineHD2300007927	28267943
23	BovineHD2300007928	28271070
23	BovineHD2300007929	28273077
23	BovineHD2300007930	28274445
23	BovineHD2300007931	28276482
23	BovineHD2300007932	28278115
23	BovineHD2300007933	28280606
23	BTA-55886-no-rs	28281915
23	BovineHD2300007934	28283075
23	BovineHD2300007935	28288210
23	BovineHD2300007936	28290987
23	BovineHD2300007937	28292297
23	BovineHD2300007938	28293679
23	BovineHD2300007939	28296493
23	BovineHD2300007940	28299628
23	BovineHD2300007941	28301056

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300007942	28302999
23	BovineHD2300007943	28304198
23	BovineHD2300007944	28305652
23	BovineHD2300007945	28306879
23	BovineHD2300007946	28309160
23	UA-IFASA-7712	28311070
23	BovineHD2300007947	28316734
23	BovineHD4100016103	28317753
23	BovineHD2300007949	28322702
23	BovineHD2300007951	28328861
23	BovineHD2300007952	28330316
23	BovineHD2300007953	28332494
23	BovineHD2300007963	28360190
23	BovineHD2300007965	28366219
23	BovineHD2300007971	28380703
23	BovineHD2300007972	28382993
23	BovineHD2300007973	28385445
23	BovineHD2300007977	28393677
23	BovineHD2300007978	28395288
23	BovineHD2300007979	28396815
23	BovineHD2300007980	28398335
23	BovineHD2300007981	28400087
23	BovineHD2300007982	28401762
23	BovineHD2300007983	28402867
23	BovineHD2300007985	28406839
23	BovineHD2300007986	28408890
23	BovineHD2300007987	28410171
23	BovineHD2300007988	28411127
23	BovineHD2300007989	28412195
23	ARS-BFGL-NGS-28555	28413350
23	BovineHD2300007993	28420236
23	BovineHD2300007994	28422804
23	BovineHD2300007996	28431174
23	BovineHD2300007998	28434257
23	BovineHD2300007999	28435313
23	BovineHD2300008001	28439058
23	BovineHD2300008002	28440539
23	BovineHD2300008003	28442281

Chromosome	SNP Name	UMD 3.1 Position
23	BTA-111017-no-rs	28444435
23	BovineHD2300008005	28448873
23	BovineHD2300008006	28450670
23	BovineHD2300008012	28469826
23	BovineHD2300008019	28486281
23	BovineHD2300008020	28487275
23	BovineHD2300015634	28494393
23	BovineHD2300008043	28561217
23	ARS-BFGL-NGS-39	28563533
23	BovineHD2300008044	28564920
23	BovineHD2300008045	28565821
23	BovineHD2300008046	28566758
23	BovineHD2300008047	28567640
23	BovineHD2300008048	28568611
23	BovineHD2300008049	28569684
23	BovineHD2300008050	28570767
23	BovineHD2300008051	28571770
23	BovineHD2300008052	28573214
23	BovineHD2300008053	28574162
23	BovineHD2300008054	28576280
23	BovineHD2300008056	28578501
23	BovineHD2300008057	28579375
23	BovineHD2300008058	28581361
23	BovineHD2300015638	28588990
23	BovineHD2300008060	28594947
23	BovineHD2300008061	28596093
23	ARS-BFGL-NGS-60100	28598059
23	BovineHD2300008062	28599086
23	BovineHD2300008063	28600222
23	BovineHD2300008064	28602332
23	BovineHD2300008065	28603103
23	BovineHD4100016104	28604139
23	BovineHD2300008066	28605081
23	BovineHD2300008067	28606252
23	BovineHD2300008068	28607959
23	BovineHD2300008069	28608693
23	BovineHD2300008070	28609602
23	BovineHD2300008071	28610641

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300008072	28611759
23	BovineHD2300008073	28613377
23	BovineHD2300008074	28614313
23	BovineHD2300008075	28615196
23	BovineHD2300008076	28616151
23	BovineHD4100016105	28617272
23	BovineHD2300008078	28619649
23	BovineHD4100016106	28620444
23	BovineHD2300008079	28621623
23	BovineHD2300008080	28622263
23	BovineHD2300008081	28622986
23	BovineHD2300008082	28625666
23	BovineHD2300008083	28626894
23	BovineHD2300008084	28628871
23	BovineHD2300008086	28630914
23	BovineHD2300008087	28631696
23	BovineHD2300008088	28632619
23	BovineHD2300008089	28633429
23	BovineHD4100016107	28634603
23	BovineHD2300008090	28635439
23	BovineHD2300008091	28636396
23	BovineHD2300008092	28637409
23	BovineHD2300008093	28638238
23	BovineHD2300008095	28642554
23	BovineHD2300008096	28643641
23	BovineHD2300008097	28645014
23	BovineHD2300008098	28647232
23	ARS-BFGL-NGS-83284	28649349
23	BovineHD2300008099	28650456
23	BovineHD2300008100	28651339
23	BovineHD2300008101	28653356
23	BovineHD2300008103	28656912
23	BovineHD2300008104	28658571
23	BovineHD2300008106	28661313
23	BovineHD2300008107	28662996
23	BovineHD2300008108	28664175
23	BovineHD2300008109	28666233
23	BovineHD2300008110	28667972

Chromosome	SNP Name	UMD 3.1 Position
23	BovineHD2300008112	28671240
23	BovineHD2300008113	28671895
23	BovineHD2300008114	28673324
23	UA-IFASA-9236	28674580
23	BovineHD2300008115	28675656
23	BovineHD4100016108	28676726
23	BovineHD2300008117	28679096
23	BovineHD2300008118	28679974

APPENDIX 2

Mizzu SNP #	SNP Name	BTA 4.0 Position
5421	ARS-BFGL-NGS-12818	26336469
14019	ARS-BFGL-NGS-4203	26359831
4731	ARS-BFGL-NGS-109142	26404894
29923	BTA-27247-no-rs	26797818
20685	ARS-BFGL-NGS-85480	26873151
22219	ARS-BFGL-NGS-96349	27014916
716	ARS-BFGL-BAC-28338	27078160
13051	ARS-BFGL-NGS-38776	27114828
19742	ARS-BFGL-NGS-79310	27162533
52187	Hapmap47328-BTA-56087	27196973
55051	Hapmap53349-ss46526376	27285019
22795	ARS-USMARC-Parent-AY937242-rs1	27286587
20171	ARS-BFGL-NGS-82024	27330080
18687	ARS-BFGL-NGS-72442	27368932
3329	ARS-BFGL-NGS-104658	27429820
7892	ARS-BFGL-NGS-2116	27471183
3656	ARS-BFGL-NGS-105633	27542578
14296	ARS-BFGL-NGS-43021	27580795
14285	ARS-BFGL-NGS-42980	27719069
22636	ARS-BFGL-NGS-99242	27745730
5149	ARS-BFGL-NGS-11887	27911886
22400	ARS-BFGL-NGS-97747	27939837
11374	ARS-BFGL-NGS-32979	27977781
6543	ARS-BFGL-NGS-16619	28007939
51974	Hapmap46836-BTA-55820	28043179
31416	BTA-55821-no-rs	28064091
31417	BTA-55853-no-rs	28112250
21417	ARS-BFGL-NGS-90570	28140134
31418	BTA-55867-no-rs	28165417
14116	ARS-BFGL-NGS-4240	28206725
1706	ARS-BFGL-NGS-100422	28268180
57703	UA-IFASA-5823	28312087
24423	ARS-BFGL-NGS-113235	28341860
51675	Hapmap44774-BTA-55887	28436700
10069	ARS-BFGL-NGS-28555	28542478

Mizzu SNP #	SNP Name	BTA 4.0 Position
16683	ARS-BFGL-NGS-60100	28752297
14385	ARS-BFGL-NGS-43333	28782231
20362	ARS-BFGL-NGS-83284	28803585
58252	UA-IFASA-9236	28828816
10970	ARS-BFGL-NGS-31638	28890490
19968	ARS-BFGL-NGS-80691	28943537
3751	ARS-BFGL-NGS-105966	29058544
49550	Hapmap40502-BTA-17475	29094169
23361	ARS-BFGL-NGS-110774	29305663
22205	ARS-BFGL-NGS-96241	29347180
22122	ARS-BFGL-NGS-95687	29368535
16662	ARS-BFGL-NGS-59981	29395930
12974	ARS-BFGL-NGS-38483	29440989
1094	ARS-BFGL-BAC-35219	29471875
4879	ARS-BFGL-NGS-109612	29745302
3224	ARS-BFGL-NGS-104394	30066821
57355	INRA-655	30091550
26457	ARS-BFGL-NGS-117913	30117027

APPENDIX 3

Common SNP List	50K BTA 4.0 Position	770K UMD 3.1 Position
ARS-BFGL-NGS-12818	26336469	25472436
ARS-BFGL-NGS-4203	26359831	25507676
ARS-BFGL-NGS-109142	26404894	25552635
BTA-27247-no-rs	26797818	26736263
ARS-BFGL-NGS-85480	26873151	26812296
ARS-BFGL-NGS-96349	27014916	26995067
ARS-BFGL-BAC-28338	27078160	27058749
ARS-BFGL-NGS-38776	27114828	27088825
Hapmap47328-BTA-56087	27196973	27215001
ARS-BFGL-NGS-72442	27368932	27383176
ARS-BFGL-NGS-104658	27429820	27444064
ARS-BFGL-NGS-2116	27471183	27485467
ARS-BFGL-NGS-105633	27542578	27545231
ARS-BFGL-NGS-43021	27580795	27583474
ARS-BFGL-NGS-99242	27745730	27644218
ARS-BFGL-NGS-11887	27911886	27776075
ARS-BFGL-NGS-97747	27939837	27804037
ARS-BFGL-NGS-32979	27977781	27841983
Hapmap46836-BTA-55820	28043179	27923154
BTA-55821-no-rs	28064091	27944066
BTA-55853-no-rs	28112250	27992880
ARS-BFGL-NGS-90570	28140134	28021047
BTA-55867-no-rs	28165417	28046330
ARS-BFGL-NGS-4240	28206725	28087630
UA-IFASA-5823	28312087	28193501
ARS-BFGL-NGS-113235	28341860	28223274
ARS-BFGL-NGS-28555	28542478	28413350
ARS-BFGL-NGS-60100	28752297	28598059
ARS-BFGL-NGS-83284	28803585	28649349
UA-IFASA-9236	28828816	28674580

APPENDIX 4

Index ID	SNP Identifier	Reason for Removal
5	HD2300008159	Unclusterable
19	HD2300008191	Unclusterable
30	HD2300008061	Unclusterable
42	HD2300007272	Unclusterable
50	HD2300007628	Unclusterable
56	HD2300007096	Unclusterable
71	HD2300007119	Unclusterable
73	HD2300007754	Unclusterable
86	HD2300007245	Unclusterable
87	HD2300008141	Unclusterable
108	HD2300007485	Unclusterable
114	HD2300007068	Unclusterable
119	HD2300007776	Unclusterable
126	HD2300007816	Unclusterable
134	HD2300007683	Unclusterable
146	HD2300008193	Unclusterable
147	HD2300008272	Unclusterable
156	HD2300016099	Unclusterable
179	HD2300007935	Unclusterable
180	HD2300008126	Unclusterable
212	HD2300008086	Unclusterable
220	HD4100016109	Unclusterable
241	HD2300007791	Unclusterable
257	HD2300007409	Unclusterable
265	HD2300007089	Unclusterable
267	HD2300008239	Unclusterable
293	HD2300007222	Unclusterable
307	HD2300007849	No Calls
312	HD2300007050	Unclusterable
353	HD2300007526	Unclusterable
355	HD2300008219	Unclusterable
367	HD2300007412	Unclusterable
388	HD2300007620	Unclusterable
403	HD2300007308	Unclusterable
413	HD2300007295	Unclusterable
427	HD2300007649	Unclusterable

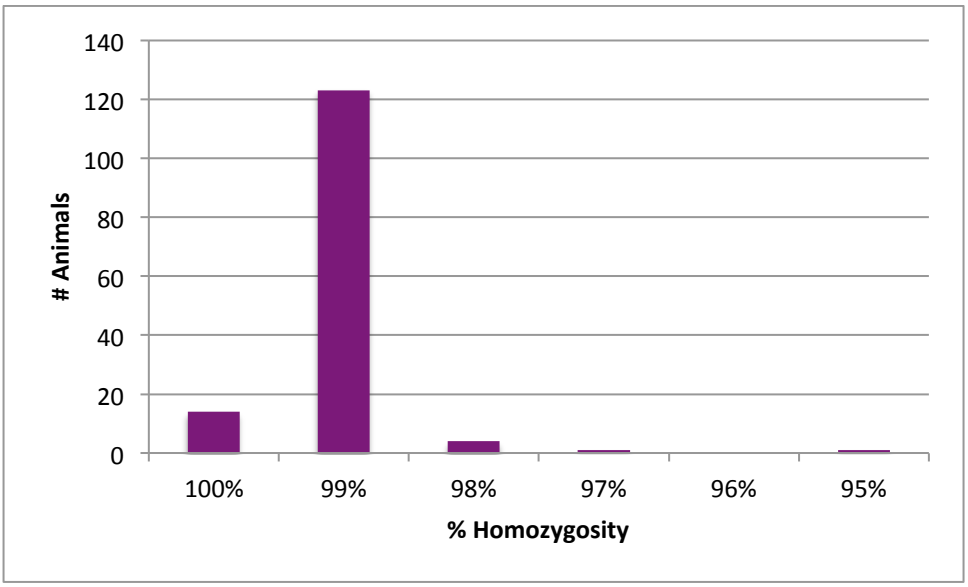
Index ID	SNP Identifier	Reason for Removal
431	HD2300007392	Unclusterable
440	HD2300007718	Unclusterable
444	HD2300007982	Low Frequency
449	HD2300015634	Unclusterable
514	HD2300007720	Unclusterable
520	ARS-BFGL-NGS-43021	Unclusterable
535	HD2300007300	Unclusterable
536	HD2300007867	Unclusterable
547	ARS-BFGL-NGS-2116	Unclusterable
549	HD2300007709	Unclusterable
555	HD2300007529	Unclusterable
557	HD2300008153	No Calls
566	HD2300007484	Unclusterable
584	HD2300007522	Unclusterable
593	HD2300007115	Unclusterable
598	HD2300007789	Unclusterable
606	HD2300007398	Unclusterable
613	HD2300008197	Unclusterable
638	HD2300007309	Unclusterable
643	HD2300007241	Unclusterable
665	HD2300008175	Unclusterable
674	ARS-BFGL-BAC-35219	Unclusterable
680	HD2300001810	Unclusterable
681	HD2300007131	Unclusterable
684	HD2300007737	Unclusterable
691	HD2300007596	Unclusterable
696	HD2300007980	Unclusterable
702	HD2300007810	Unclusterable
714	HD2300007918	Unclusterable
723	HD2300007258	Unclusterable
735	HD2300007575	Unclusterable
774	HD2300007977	Unclusterable
776	HD2300007686	Unclusterable
796	HD2300007461	Unclusterable
817	HD2300008078	Unclusterable
818	HD2300008117	Unclusterable
827	HD2300008210	Unclusterable
844	HD2300007263	Unclusterable

Index ID	SNP Identifier	Reason for Removal
853	HD2300007504	Unclusterable
860	HD2300007868	Unclusterable
864	HD2300008064	No Calls

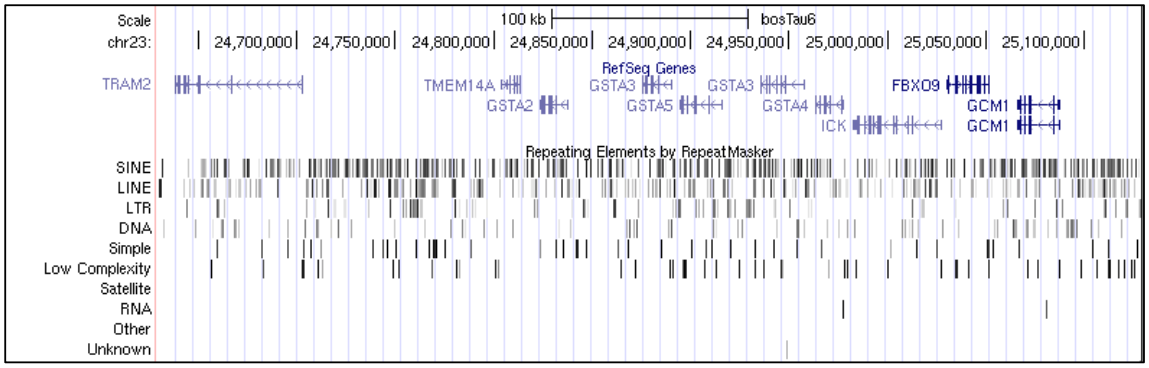
APPENDIX 5

Attached file.

(a)



(b)



Appendix 6. (a) Numbers of animals homozygous at greater than 95% of SNPs in BoLA. (b) Genomic location containing 230 SNPs in the class IIa region, that contained the majority of heterozygous SNPs from paired highly similar paired haplotypes in animals that were less than 95% homozygous.