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DEFINITION OF BOVINE LEUKOCYTE ANTIGEN DIVERSITY AND PEPTIDE
BINDING PROFILES FOR EPITOPE DISCOVERY

A Dissertation Presented

by

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ABSTRACT

The goal of the work presented herein was to further our understanding of Bovine Leukocyte Antigen (BoLA) class I diversity of Holstein cattle and develop tools to measure class I restricted T cell responses to intracellular pathogens such as foot and mouth disease virus (FMDV) following vaccination. BoLA is a highly polymorphic gene region that allows the bovine immune system to differentiate pathogen-infected cells from healthy cells. Immune surveillance by CD8⁺ T cells plays an important role in clearing viral infections. These CD8⁺ T cells recognize BoLA class I molecules bearing epitopes (antigenic peptides) of intracellular origin in their peptide binding groove. Polymorphisms in the peptide binding region of class I molecules determine affinity of peptide binding and stability during antigen presentation. Different antigen peptide motifs are associated with specific genetic sequences of class I molecules. In order to better understand the adaptive immune response mediated by BoLA molecules, technologies from human medicine such as high-throughput sequencing, biochemical affinity and stability assays, tetramers and IFN- γ ELISpot assays could be applied. Therefore, it was hypothesized that we can translate these technologies from the study of human T cell responses to the study of cattle immunity.

The first objective was to establish a comprehensive method for genotyping BoLA of Holstein cattle by using Illumina MiSeq, Sanger sequencing and polymerase chain reaction sequence-specific primers (PCR-SSP) (See Chapter 2). This is an important first step in order to study the BoLA restricted immune responses following FMDV vaccination. The second objective was to define the FMDV capsid protein peptide repertoire bound by BoLA class I molecules using bioinformatics and biochemical affinity and stability assays to facilitate the identification of T cell epitopes (See Chapter 3). The third objective was to demonstrate clonal T cell expansion for specific epitope polypeptides using *ex-vivo* multi-color flow cytometric MHC-epitope complexes (tetramers), followed by IFN- γ production measured by an ELISpot assay to quantify and define the antigen specific response of Holstein cattle to FMDV vaccination (see Chapter 4). In this, my dissertation studies aimed to improve our understanding of the BoLA class I restricted T-cell responses to candidate FMDV vaccines in Holstein cattle. In this manner, my research will improve animal health through the production of assays for characterizing the bovine immune response to intracellular pathogens and enhance vaccine design leading to improved biologicals to protect cattle from devastating infectious diseases.

CITATIONS

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1. CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1. Introduction

Cattle are one of the most economically important livestock species in the United States and worldwide. Cattle were first domesticated among migratory human populations approximately 10,000 years ago (Ensminger 1993). They are not only a valuable source of milk, meat, and hide, but also can be used as draft animals. In 2015, the global cattle population provided 63 million tonnes of meat and 126 million tonnes of skimmed cow milk (FAOSTAT 2013). The continual rise in the world's population is estimated to reach 9 billion people by the year 2040 (Bureau 2015). This increase will demand a need for animal protein; cattle are import source for this protein. Infectious diseases such as foot and mouth disease virus (FMDV) are a major economic constraint on cattle productivity and a threat to food safety. Although cattle are primarily bred for their milk and meat production, disease resistance is a quality strongly desired by most producers.

The major histocompatibility complex (MHC) genes play a seminal role in the immune response of mammalian species, such as cattle. MHC is a set of genes that bind pathogen-derived peptides and presents them on the surface of nucleated cells for recognition by specialized T cells. As a result of pathogen recognition via MHC presentation, T cells and B cells are activated, expanded and differentiated into effector cells, which help defend the body against pathogens. One of the key features of the MHC gene region is that it is highly polymorphic. This high polymorphism allows individuals

with diverse MHC genes bind and recognize peptides derived from numerous different pathogens (Sette and Sidney 1999).

1.2. MHC Discovery

The MHC was first described in mice in the 1940's by George Snell and Peter Gorer (Gorer and Schutze 1938; Snell 1948). Initially, the MHC was known for its role in immune acceptance or rejection of transplant tissues. It was later discovered that immune surveillance by CD8+ or CD4+ T cells begins with the recognition of foreign or self-antigens presented at the cell surface by MHC class I or II molecules (Bjorkman and Parham 1990; Maffei et al. 1997). The most selective event in antigen presentation is the binding of the peptide to the MHC molecule (Yewdell and Bennink 1999). Selection of T cells that are self-MHC restricted occurs in the thymus during T cell maturation (Goldrath and Bevan 1999). Those T cells that do not recognize self-MHC die and only the T cells that express antigen receptors for specific peptides bound to self MHC are appropriately stimulated and survive (Goldrath and Bevan 1999). CD8+ T cells are MHC class I restricted and CD4+ T cells are class II MHC restricted.

1.3. MHC Molecules

1.3.1. MHC Class I

MHC class I molecules are a trimeric complex composed of a heavy α chain (45 kDa) noncovalently associated with a β_2 -microglobulin (β_2 M) light chain, (12 kDa) and exogenous peptide (Bjorkman and Parham 1990; Grey et al. 1973) (Figure 1-1). The α chain is polymorphic and encoded by the MHC gene. It is composed of three

extracellular domains, $\alpha 1$, $\alpha 2$, $\alpha 3$, a hydrophobic transmembrane domain and hydrophilic cytoplasmic tail (Flutter and Gao 2004). The hypervariable $\alpha 1$ and $\alpha 2$ domains form the peptide binding groove of class I molecules. The other polypeptide chain that composes the MHC molecule is $\beta 2$ -microglobulin, which is encoded by the $\beta 2M$ gene. The $\alpha 3$ and $\beta 2M$ are more conserved and are homologous to immunoglobulin domains (Becker and Reeke 1985; Bjorkman and Parham 1990; Grey et al. 1973).

In cattle the MHC is called the bovine leukocyte antigen (BoLA) gene locus. The existence of BoLA was established over 35 years ago (Spooner et al. 1979). Similar to humans and mice, cattle have three MHC gene classes: class I, II and III. BoLA class I and II both play a role in antigen presentation, whereas the function of BoLA class III has been associated with components of the complement system (Ellis and Hammond 2014; McShane et al. 2001). In contrast to humans, who have a fixed complement of three classical class I genes, human leukocyte antigen (HLA)-A, B and C, cattle express a variable number of genes expressed in a range of different combinations. To date, six BoLA class I genes have been identified. Typically 1-3 of these genes are expressed in variable combinations to compose a variety of haplotypes. 13 haplotype configurations have been identified, primarily from European cattle populations (Codner et al. 2012a). Understanding the evolutionary history of the MHC regions in Cetariodactyls, such as cattle will provide insights into predicting and manipulating the T cell responses in these species. Over 13 million years ago there have been several evolutionary events that have contributed the divergence of the *Bovinae* MHC subfamily (Ellis and Ballingall 1999; Holmes et al. 2003). These events are attributed to large-scale gene block duplications

and selective pressures, which contribute to the overall diversity in the BoLA class I genes (Ellis and Ballingall 1999). The BoLA variants have important roles in biological functions, such as influencing the immune response and susceptibility to infectious diseases (Ellis and Ballingall 1999; Ellis and Hammond 2014; Holmes et al. 2003).

The overall genomic organization of the MHC in cattle differs slightly from that in mice and humans. The BoLA region in cattle is split into three gene classes, however BoLA class II region is further divided into class IIa and class IIb (Sharif et al. 1998). The overall size of the BoLA class I gene region is approximately 400 Kb and is located on chromosome 23 (Di Palma et al. 2002). All validated BoLA allele sequences are publicly available in the Immuno-Polymorphism Database (IPD-MHC) (Robinson et al. 2010).

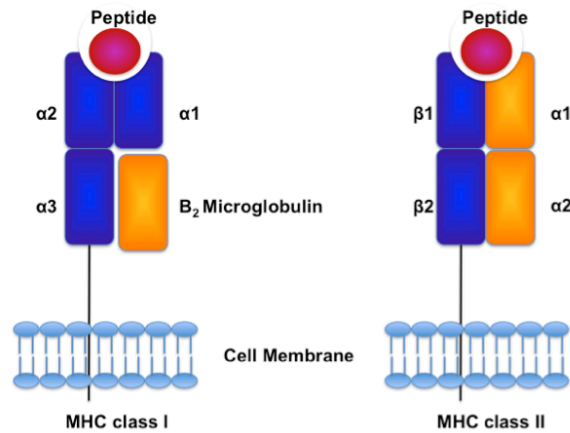


Figure 1-1: Simplified structure of MHC class I and II molecules. Figure made by author.

1.3.2. Processing of Cytosolic Antigens for MHC Class I Presentation

Pathogen-derived polypeptides between 8-12 amino acids in length are presented by cell surface MHC class I molecules to T cells. For example, during FMDV viral infection of a host cell, FMDV antigens found in the cytosol or nucleus are processed and presented via the endogenous pathway within nucleated cells (Maffei et al. 1997). In the endogenous pathway, proteins present in the cytosol or nucleus are degraded by the proteasome into short peptides. A small polypeptide, ubiquitin, targets these proteins for proteasomal degradation by covalent linkage. Ubiquitinated proteins are unfolded and “threaded” through the proteasome. LMP-2 and LMP-7 are two catalytic subunits present in 1500 kD proteasomes that are encoded by MHC genes and are important for generating class I peptides (Yewdell et al. 1994). The ATP-dependent transport of the resulting peptides from the cytosol into the lumen of the endoplasmic reticulum (ER) is accomplished by the transporter associated with antigen processing (TAP) (Maffei et al. 1997). Once on the luminal side of the ER, the TAP protein is noncovalently attached to newly synthesized MHC class I molecules by tapasin, a linker protein (Peaper and Cresswell 2008). Upon antigen sensitization T cells can cause an upregulation of IFN- γ , which increases synthesis of LMP-2 and LMP-7. This in turn enhances antigen presentation, as well as TAP, thus enhancing killing of virus-infected cells (Yewdell et al. 1994).

Before the translocated peptide can bind to an MHC molecule, the MHC molecule must first be properly assembled. ER chaperone proteins, such as calnexin and calreticulin, assist with the correct folding of the α chains. β_2M is synthesized in the ER,

stabilizing the class I α chains. The peptide is trimmed to 8-12 amino acids in length by the ER-resident aminopeptidase (ERAP) for correct loading into MHC class I. Once the peptide is able to bind in the cleft of the MHC class I molecule, tapasin is released and the peptide-MHC complexes are subsequently trafficked through the Golgi. They are then glycosylated and transported in secretory vesicles, which then fuse with the cellular membrane (Figure 1-2). Presentation of the MHC-peptide complexes on the surface of the infected cells permits interaction with T cell receptors (TCRs) expressed on the surface of CD8⁺ T cells. T cell receptors demonstrate specificity to individual MHC-peptide complexes; thus, specificity of the immune response to specific peptides is regulated at both the level of binding of the expressed MHC-peptide complex to complementary receptors of T cell populations and the binding of pathogen-derived polypeptide in the binding cleft of the MHC molecules.

1.3.3. MHC Class II

MHC class II molecules are heterodimers composed of 33 kDa α -chain and 28 kDa β -chain associated by noncovalent interactions (Figure 1-1) (Schafer et al. 1995). Each chain is composed of two α or two β domains. Similar to MHC class I molecules, MHC class II molecules contain transmembrane and cytoplasmic domains (Figure 1-1). The groove between β 1 and α 1 domains is a highly polymorphic peptide binding groove.

In humans, MHC class II molecules are encoded by three polymorphic genes: HLA-DR, HLA-DQ and HLA-DP (Neefjes et al. 2011). Cattle express only two BoLA class II proteins, DR and DQ (Norimine and Brown 2005). The BoLA-DQ molecules are duplicated and generate diversity through inter-haplotype and intra-haplotype pairing

(Norimine and Brown 2005). The DR α chain in humans and cattle is monomorphic. The only source of diversity in DR molecules is from the polymorphic DR β chain (Norimine and Brown 2005). In cattle only the DR β 3 gene is functional.

1.3.4. Processing of Endocytosed Antigens for MHC Class II Presentation

MHC class II molecules are primarily expressed by antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages and B cells (Neeffjes et al. 2011). APCs endocytose and process protein antigens through the endosomal pathway, proteins are continuously degraded and cycled through early and late endosomes and lastly into lysosomes. Within these compartments the internalized proteins are exposed to increasingly acidic conditions and are degraded enzymatically by proteases, such as cathepsins, to produce peptides (Roche and Furuta 2015).

MHC Class II molecules are synthesized in the ER. A polypeptide chaperone protein termed the invariant chain (I_i) binds to the peptide binding cleft of the MHC molecule in the ER. I_i promotes folding and assembly of class II molecules and facilitates MHC transfer to the endosomal pathway (Roche and Furuta 2015). Once the I_i -MHC class II heterotrimer is transported through the Golgi to an endosome called the MHC class II compartment (MIIC), cathepsin S degrades the I_i , leaving the class II-associated I_i peptide (CLIP) fragment in the peptide binding groove of the MHC class II dimer (Roche and Furuta 2015). The CLIP fragment is later exchanged for an antigenic peptide with the help of chaperone protein HLA-DM (Neeffjes et al. 2011). The peptide-MHC complexes

are then transported to the cell surface, where they are displayed for recognition by CD4+ T helper cells (Figure 1-2).

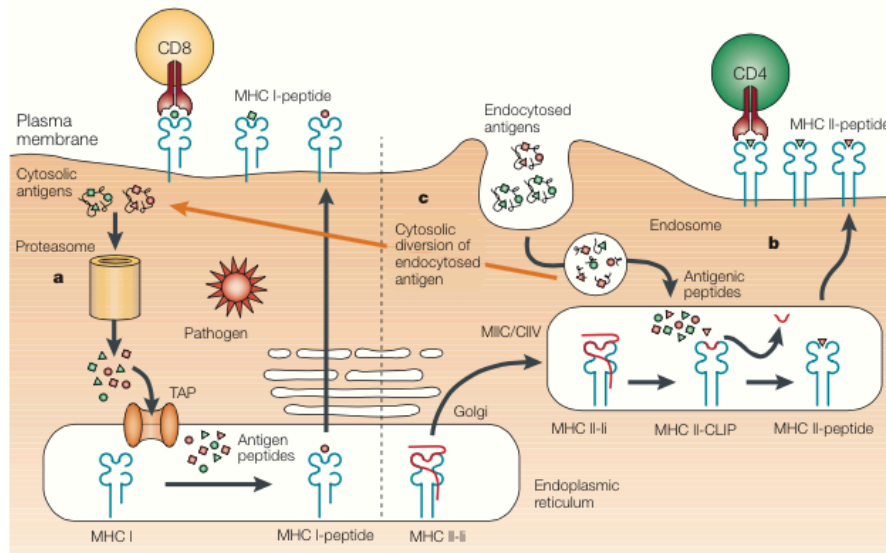


Figure 1-2: Schematic diagram of antigen presentation pathways by MHC class I and II (Heath and Carbone 2001).

1.3.5. Non-classical MHC Molecules

The MHC class I and II regions encode a number of genes termed ‘non-classical’ (Birch et al. 2008). Non-classical MHC molecules are non-polymorphic, encode heavy chains with truncated cytoplasmic domains and are not ubiquitously expressed (Birch et al. 2008). In humans, HLA-E, -F and -G constitute the non-classical class I genes. Non-classical molecules in humans and mice have been shown to play a role in immune evasion, natural killer (NK) cell activation and reproduction (Hofstetter et al. 2011). In cattle there are five non-classical genes (NC1-5), composed of 19 alleles in IPD-MHC. Previous transcription studies found that NC1 is ubiquitously expressed and NC2-5 expression is much more variable (Codner et al. 2012a; Ellis and Hammond

2014). NC1 is found adjacent to the classical class I genes on chromosome 23, whereas NC2-4 are located 500 Kb centromeric to the cluster of classical class I genes, NC5 has not been mapped (Birch et al. 2008). The functional importance of these non-classical MHC class I genes in bovine is not well understood.

1.4. Polymorphism & Generation of MHC Diversity

1.4.1. Genetic Mechanisms Contributing to MHC Diversity

The MHC contains the most polymorphic genes described in vertebrates (Piertney and Oliver 2006). As of July 2015, there were 3,192 HLA-A, 3,977 HLA-B and 2,740 HLA-C known alleles (<http://hla.alleles.org/nomenclature/stats.html>). In cattle there are presently 114 BoLA class I alleles including 95 classical alleles and 19 non-classical alleles in IPD-MHC. In humans and cattle, MHC polymorphisms arise from point mutations, gene duplication or deletion and intra-locus recombination (Codner et al. 2012a; Codner et al. 2012b; Ellis and Hammond 2014; Parham et al. 1995). The majority of the MHC variation lies within the peptide binding region of class I and II molecules (Parham et al. 1995). The maintenance and generation of MHC diversity is quite complex; however, it seems the high level of diversity ensures a broad range of immune responses to a variety of different pathogens.

1.4.2. Selection Mechanisms of MHC

There are two main models that contribute to MHC polymorphism: disease-based and reproductive mechanisms. Disease based models consist of the heterozygote advantage theory, negative frequency dependent selection hypothesis and fluctuating

selection theory. The heterozygote advantage theory was first proposed by Doherty and Zinkernagel in 1975 (Doherty and Zinkernagel 1975). This hypothesis states that within a given population where individuals are exposed to various pathogens, heterozygotes are able to present more antigens to T cells than homozygotes, thus heterozygotes are more resistant to pathogens than homozygotes (Bernatchez and Landry 2003; Doherty and Zinkernagel 1975). Another disease-based model is the negative frequency dependent selection hypothesis or rare allele advantage, proposed by Clarke and Kirby in 1966 (Bernatchez and Landry 2003; Clarke and Kirby 1966). This hypothesis proposes that new or rare alleles incur a selective advantage when a novel pathogen strain arises. Therefore, the fitness advantage of a rare allele results in positive selection and causes an increase in phenotype, while the more common haplotype will decrease in frequency (Codner 2010). The last disease based model is the fluctuating selection theory. This theory states that MHC diversity is maintained by spatial and temporal heterogeneity in the type and abundance of pathogens present and that MHC selection is directional and that pathogen flux is determined by the environment (Hill et al. 1991; Spurgin and Richardson 2010).

Reproductive mechanisms for MHC polymorphism include the disassortative mating preferences and inbreeding avoidance theories. The disassortative mating theory states that disease-based fitness differences between MHC genotypes would lead to reproductive mechanisms favoring high-fitness MHC heterozygotic offspring (Bernatchez and Landry 2003; Piertney and Oliver 2006). The inbreeding avoidance hypothesis suggests that since the MHC loci is polymorphic, individuals sharing the same

MHC alleles are likely to be related. Therefore, matings among related individuals is discouraged in order to prevent recessive deleterious mutations (Bernatchez and Landry 2003). It is also proposed that specific odors produced by an individual is a potential marker for relatedness, hence this could be a cue to avoid inbreeding (Bernatchez and Landry 2003; Piertney and Oliver 2006).

1.4.3. Characterizing Polymorphisms of the BoLA Class I Region

Occurrence of BoLA alleles in a cattle population exposed to pathogens such as FMDV can provide insights on particular epitopes, which can ultimately aid in vaccine design. Polymorphisms at BoLA class I loci were initially detected using serology (Ellis et al. 1998; Spooner et al. 1979). However, molecular techniques such as cloning and sequence specific PCR (PCR-SSP) have demonstrated increased BoLA allelic diversity within serologically defined haplotypes and improved discriminatory power among BoLA allelic polymorphisms (Babiuk et al. 2007; Birch et al. 2006; Ellis et al. 1998). This additional data has improved our understanding of BoLA molecules associated with serologically defined haplotypes and has allowed for discrimination between BoLA molecules (Babiuk et al. 2007; Birch et al. 2006; Ellis et al. 1998). As such, improved methods for identifying existing and new BoLA alleles are needed. Recent advances in next generation sequencing (NGS) technologies allow for the sequencing of highly polymorphic regions of the genome, such as the MHC locus. These high-throughput methodologies have been applied to MHC class I typing for several species, such as humans, nonhuman primates, and swine, but have been less commonly used in cattle. Knowledge of the degree of polymorphism and expression levels of specific BoLA

alleles is fundamental to understanding the functional importance of a particular locus.

Comparison of different BoLA class I typing methods is shown in Table 1-1.

Table 1-1: Comparison of BoLA typing methods

Typing Assays for BoLA Class I	Strengths	Limitations	References
Serology	<ul style="list-style-type: none"> •High Throughput •Inexpensive 	<ul style="list-style-type: none"> •Lacks specificity at allelic level •Limited availability of antibodies •Requires large volumes of blood •Requires viable lymphocytes •Difficult to find good antisera for rarer antigens in different populations •Fresh samples 	(Caldwell et al. 1977; Spooner et al. 1979)
Sequence Specific Polymerase Chain Reaction (SSP-PCR)	<ul style="list-style-type: none"> •Rapid •Relatively inexpensive 	<ul style="list-style-type: none"> •No new allele discovery •False Positives •False Negatives •Negative results are ambiguous 	(Ellis et al. 1998)
Isoelectric focusing (IEF)	<ul style="list-style-type: none"> •High Resolution •Fills in serological blanks •Provided support for serologically defined haplotypes •Distinguishes heterozygotes from homozygotes •Detects subtypes and splits 	<ul style="list-style-type: none"> •Labor Intensive •Expensive •Gel to Gel variation •Need expertise in the assay 	(Davies CJ 1994; Joosten et al. 1991; Oliver et al. 1989)
Restriction fragment length polymorphism (RFLP)	<ul style="list-style-type: none"> •Able to identify new alleles •Able to resolve ambiguous heterozygous combinations •Fresh samples not needed •No 'blank' alleles •Inexpensive •Multiple enzymes are available 	<ul style="list-style-type: none"> •Labor intensive •Only detects polymorphisms at restriction sites •Does not tell identity of polymorphism, just location 	(Oliver et al. 1989) (Lindberg and Andersson 1988)
Sanger Sequencing (from clones)	<ul style="list-style-type: none"> •Identifies new alleles 	<ul style="list-style-type: none"> •Time consuming •Expensive •Primer specificity 	(Ellis et al. 1998)
Reference Strand Mediated	<ul style="list-style-type: none"> •Identify new alleles are present 	<ul style="list-style-type: none"> •Does not tell identity of polymorphism 	(Arguello and Madrigal 1999) (Birch et al. 2006)

Conformation (RSCA)	<ul style="list-style-type: none"> •Potentially detects polymorphisms along whole gene •Comparing mobility values to previously sequenced alleles 	•Cost	(Codner 2010)
Pyrosequencing 454	<ul style="list-style-type: none"> •High Throughput •Up to 1kb read length 	<ul style="list-style-type: none"> •Expensive •AT rich genes don't sequence well Polyhomodimers interfere with sequence reads •Sequence assembly •Primer specificity •Outdated Platform 	(Babik et al. 2012)
Illumina MiSeq	<ul style="list-style-type: none"> •High Throughput •Up to 600 bp read length 	<ul style="list-style-type: none"> •Short read length •Sequence assembly •Primer specificity 	

1.5. Peptide binding & T cell epitopes

1.5.1. Peptide binding

Despite the fact that peptide binding to MHC class I molecules is the most selective step in determining immunogenicity of antigens from intracellular pathogens such as viruses (Yewdell and Bennink 1999); the diversity of peptide-BoLA binding specificity has only been characterized for a limited number of BoLA molecules (Hansen et al. 2014). Understanding peptide-BoLA class I binding specificities may facilitate development of vaccines or reagents for quantifying the adaptive immune response to intracellular pathogens, such as foot and mouth disease virus (FMDV). Polymorphisms in the peptide binding region of class I molecules determine peptide binding affinity and stability during antigen presentation, which can be measured by biochemical assays (See Chapter 2). The peptides that bind to class I molecules often share common features (i.e., motifs); this is a function of key amino acid residues in the binding pockets of the MHC

molecules. Typically positions 2, 3, and 9 are the critical anchor positions required for peptide binding (Rammensee et al. 1995). The antigenic peptides (epitopes) presented in the context of MHC class I molecules on the surface of virus-infected cells or activated antigen presenting dendritic cells are recognized by TCRs on CD8⁺ T cells.

1.5.2. Characterization of T cells

T cells develop in the thymus from a common lymphoid progenitor and are defined by expression of a TCR. The TCR is responsible for recognizing antigenic peptides presented by MHC. T cell activation is controlled by a) local antigen concentration, b) the threshold number of engaged TCRs as a fraction of a cell's total TCRs, c) the ability of the cell to engage its TCR sequentially and d) the availability of co-stimulation (Rothenberg 1996). T cells need two extracellular signals to initiate proliferation and differentiation into activated cells known as effector cells. The first signal is the binding of peptide-MHC (pMHC) complexes on the surface of APC to the TCR. The second signal is provided by co-stimulators such as CD28 and CD80, which trigger T cell activation (Malissen et al. 2014). T cell accessory molecules that aid with signal transduction are CD3, ζ (zeta), CD4 and CD8 (Abbas 2007).

CD4⁺ and CD8⁺ T cells are activated in different ways. Activation of CD4⁺ T cells requires the presence of antigen in the same lymphoid tissue as the CD4⁺ T cell. This is possible due to the circulation of naïve T cells from the blood to the lymphoid tissue. A MHC class II molecule of an APC, typically a dendritic cell, must present the antigen in order to trigger activation of T cells. CD4⁺ T cells also require co-stimulation by IL-2 and B7 proteins to become activated. Activation of CD8⁺ T cells also requires

antigen recognition, but by MHC class I-associated peptide antigens (Abbas 2007). In addition, helper CD4⁺ T cells can stimulate activation of naïve CD8⁺ T cells and can promote their differentiation into functional CTLs by inducing cytokine expression by APCs. For example, activated CD4⁺ T cells express the CD40 ligand, which binds to a CD40 receptor on an APC, causing stimulation of CD8⁺ T cells (Abbas 2007). Once CTLs are activated they can produce perforin, granzymes, IFN- γ , lymphotoxin and tumor necrosis factor (TNF), triggering cell death and inflammation (Abbas 2007).

The affinity of the TCR for pMHC complexes is much lower than that of antibodies (produced by B lymphocytes and not discussed in this thesis). The dissociation constant (K_d) of TCR interactions with peptide-MHC complexes ranges from 10^{-5} to 10^{-7} M (Abbas 2007). This low antigen-binding affinity creates the need for adhesion molecules (integrins) to stabilize the binding between the T cell and the APC. The interaction between the T cell and the APC initially occurs in the plasma membrane of cells containing glycosphingolipids and protein receptors (lipid rafts), which form an immunological synapse. Integrins also mediate migration of T cells in response to antigen through a series of intracellular signaling events such as: phosphorylation of TCR chains, triggering of protein tyrosine kinases and activation of enzymes such as ERK, JNK and PKC lead to the upregulation of transcription factors (NFAT, AP-1 and NF- κ B). These transcription factors act to induce specific gene transcription, leading to differentiation, proliferation and effector actions of T cells (Huse 2009).

There are three main stages of T cell responses. The first stage is initiated in the lymphoid tissues; it is known as the expansion phase (Kaech et al. 2002). In this stage,

once a naïve T cell encounters an antigen, it proliferates and differentiates into an effector T cell (Kaech et al. 2002). Once the effector T cells have successfully cleared the pathogen, the majority (>90%) of the remaining effector T cells die; this is the second stage, known as the contraction or death period (Kaech et al. 2002). Lastly, the remaining T cells can enter the memory phase, where they become stabilized and are maintained for long periods of time (Kaech et al. 2002).

Activated mature dendritic cells are efficient activators of T cells. During the first 24 hours after T cells have been activated, CD4⁺ and CD8⁺ T cells prepare for clonal expansion triggered by IL-2. CD8⁺ T cell division begins 6-8 hours after initial stimulation and CD4⁺ T cell division occurs 36-48 hours after the initial stimulation. This leads to differentiation of the T cells into effector cells or memory cells. In the effector phase of the response, CD4⁺ T cells secrete cytokines, activating macrophages and B cells. CD8⁺ T cells respond by killing the infected cell thru mechanisms such as perforin, granzyme and Fas ligand pathways (Groscurth and Filgueira 1998).

1.5.3. Detection of Antigen-Specific CD8⁺ T cell Responses

It is well established that CD8⁺ T cells play an important role in clearing many intracellular pathogens. Recently there has been increased interest in developing therapeutic vaccines capable of eliciting or enhancing virus-specific CD8⁺ T cell responses. Human immunodeficiency virus (HIV), hepatitis C virus (HCV), most herpes viruses (HSV) and foot and mouth disease virus (FMDV) are examples of pathogens that can be resistant to neutralization by antibodies; therefore, T cells play a critical role in

clearance and protecting the host from these pathogens (Borrego et al. 2006; Woolard and Kumaraguru 2010).

Great progress has been made over the last decade to address the lack of accurate and quantitative assays to detect specific T cell responses (Hadrup and Schumacher 2010). A few of the current methods of high-throughput peptide-MHC (pMHC) based methods available for CD8⁺ T cell detection include ELISpot assays, tetramers and MHC microarrays (Hadrup and Schumacher 2010). ELISpot and tetramers were used in chapter 3 of this dissertation to assess CD8⁺ T cell responses following FMDV vaccination in bovine. These high-throughput methodologies are important for T cell epitope identification, monitoring and function. In combination with these technologies by using a reverse immunology approach (*in silico* epitope predictions from the sequence of a target gene of interest), is changing the way to verify the identified MHC-ligands recognized by T cells (Hadrup and Schumacher 2010). The reverse immunology approach has been used in bacterial and viral pathogens, such as meningococcus B, human immunodeficiency virus, yellow fever virus, West Nile virus, dengue virus, herpes virus, and hepatitis C virus (Bruno et al. 2015; Rappuoli 2001). This novel approach has not yet specifically been applied to FMDV in cattle.

1.5.4. *IFN- γ ELISpot Assay*

Antigen specific T cells occur in relatively low frequencies (1/100,000-1,000,000), which makes identifying them a challenge (Kalyuzhny 2012). The ELISpot assay is a highly sensitive technique to detect single bioactive molecule (e.g., cytokine) producing cells. If one million PBMCs are plated per well, the lower detection limit of

the assay is 0.0001%, which exceeds the detection limit of intracellular cytokine staining and tetramers (Kalyuzhny 2012). The ELISpot assay is the gold standard of detecting the frequency and function of antigen-specific CD8⁺ T cell responses (Malyguine et al. 2012). The use of peptide pools consisting of overlapping peptides facilitates high-throughput screening, which is an advantage of the ELISpot assay.

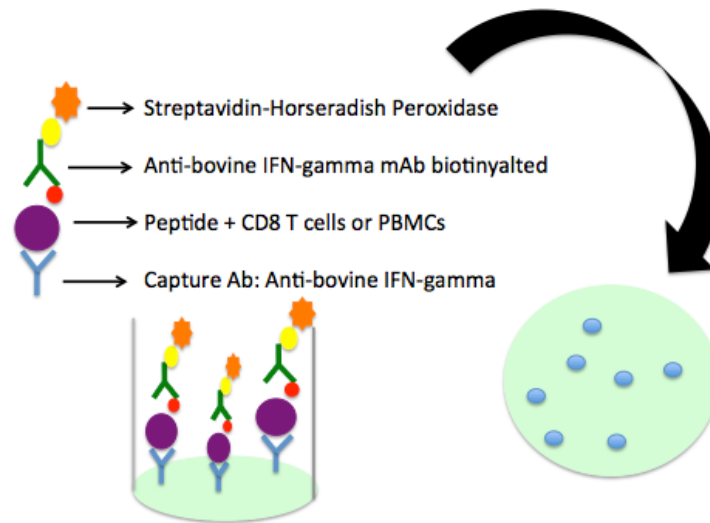


Figure 1-3: Illustration of IFN- γ ELISpot assay used in this study. Figure made by author.

For example, an IFN- γ ELISpot assay was used to detect antigen-specific MHC restricted CD8⁺ T cell responses following FMDV infection or vaccination (Guzman et al. 2010; Guzman et al. 2008). In the more recent study, they demonstrated induction of IFN- γ release by CD8⁺ T cells in response to peptide pools, which contained 15 amino acids overlapping by 10 amino acids, spanning the P1-2A protein of FMDV serotype O UKG/35/2001 (Guzman et al. 2010). After further testing of individual peptides in a positive peptide pool, one nonamer peptide (YYFADLEVA) produced a statistically significant number of IFN- γ producing CD8⁺ T cells. A schematic of the IFN- γ ELISpot

used in this thesis is outlined in Figure 1-3. Numerous other studies have used IFN- γ ELISpot assays to quantify the cellular immune responses to pathogens such as arenavirus, vaccinia virus, human immunodeficiency virus, cancer, varicella-zoster virus and bovine tuberculosis (Assarsson et al. 2007; Botten et al. 2010; Malyguine et al. 2012; Smith et al. 2001; Streeck et al. 2009; Vordermeier and Whelan 2012). The importance of cell-mediated immunity in protection against the pathogens listed above emphasizes the need for highly sensitive detection methods.

1.5.5. MHC Tetramer Technology

The recent development of tetramer technology has provided new tools for the studies of MHC restricted CD8⁺ T cell responses. Tetramer technology was developed to address the weak interaction (dissociation constants of 10^{-6} M or less) between the TCR and its ligand (Constantin et al. 2002). An MHC tetramer is a complex of four MHC class I or II glycoproteins loaded with peptide, bound to a fluorescently labeled streptavidin molecule, as visualized in Figure 1-4 (Altman et al. 1996; Klenerman et al. 2002).

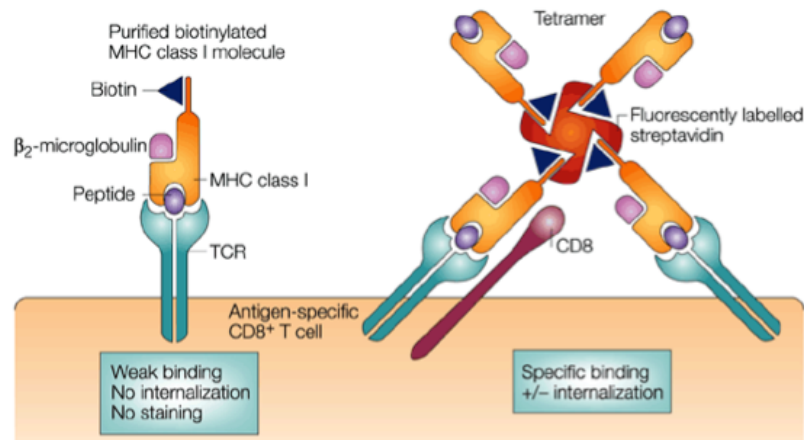


Figure 1-4: Simplified schematic of MHC class I tetramer (Klenerman et al. 2002).

The MHC tetrameric complex binds the TCR with greater avidity than a single TCR-peptide MHC interaction. The tetramers bind to T cells that are specific for both the MHC type and the peptide used in the tetramer. Antigen-specific T cells from immunized and challenged animals can be identified by tetramer staining. MHC class II tetramers in cattle have been used to enumerate and track T cells during infection or vaccination by *Anaplasma marginale* and *Babesia bovis* (Norimine and Brown 2005; Norimine et al. 2006). In swine, Patch et al., (2011) demonstrated FMDV-specific CTL killing and confirmation by MHC class I tetramer staining in response to vaccination against FMDV. More recently, Svitek et al., (2014) created and validated the first use of pMHC class I tetramers in bovine to rapidly identify *Theileria parva* specific CTL responses. There is a need to expand the BoLA tetramer technology to other molecules and epitopes, as this will advance the analysis of antigen-specific T cells, thus making it possible to better characterize the bovine cellular immune responses.

1.6. Foot and Mouth Disease

Foot-and-mouth disease (FMD) is a disease with severe agricultural and economic implications of cattle and other cloven-hoofed animals (Grubman and Baxt 2004; Lee 2010). The causative agent of FMD is a small (8.5kb), plus-sense, ssRNA virus, which belongs to the *Aphthovirus* genus of the family *Picornaviridae* (Belsham 1993). There are seven major serotypes: O, A, C, Asia 1, and South African Territories (SAT) serotypes SAT 1, SAT 2, SAT 3. Numerous subtypes occur within each serotype, reflecting significant genetic variability, which is a problem for vaccine design (Knowles and Samuel 2003).

1.6.1. Impacts of FMDV

Over past two decades, formally FMD-free countries have seen outbreaks that have raised awareness of the susceptibility of livestock and how rapidly the disease can spread (Fevre et al. 2006; Thompson et al. 2002). In 2001 an epidemic of FMD in England caused 2,030 outbreaks to occur among livestock and 15 suspected cases of human FMD infection were reported. As a result of this epidemic over 4 million animals were slaughtered and the UK economy suffered a financial loss of 12-14 billion US dollars (Scudamore and Harris 2002; Thompson et al. 2002). All 15 human cases were proven negative by reverse-transcription-polymerase chain reaction (RT-PCR) (Turbitt 2001).

Outbreaks of FMD continue to be a problem worldwide. In 2013-14 there were 974 outbreaks of FMD reported to the World Organization for Animal Health (OIE) from 18 different countries (Agriculture 2015). Approximately 70% of these outbreaks have

been resolved according to reports submitted to the OIE (Agriculture 2015). The world map describes the prevalence of FMD based on the reports submitted to OIE (Figure 1-5).

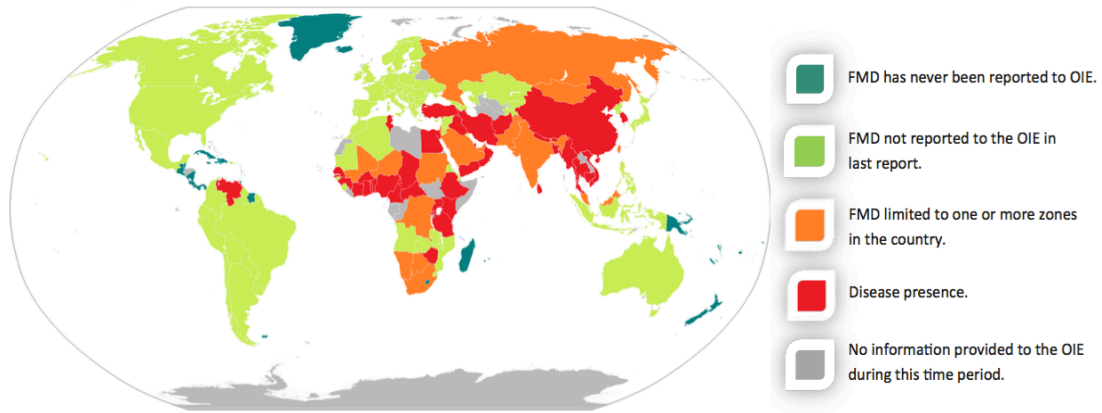


Figure 1-5: Outbreaks reported to OIE during 2013-14 (Agriculture 2015).

As shown in figure 1-5, FMD continues to be a threat to the world’s agricultural economy. Although the last outbreak of FMDV in the United States was in 1929, it is estimated that an outbreak in California alone, would have an economic impact of \$2.3-\$69.0 billion dollars (Carpenter et al. 2011).

1.6.2. Clinical Signs of Disease and Pathogenesis

FMDV is a systemic disease of domestic and wild cloven-hoofed animal species such as: cattle, swine, sheep, goats, buffaloes and camels (Alexandersen and Mowat 2005). FMD in cattle is characterized by fever (above 104°F), excessive salivation, lameness, lesions on the tongue, feet and snout, decreased milk production and general morbidity (Alexandersen and Mowat 2005; Klein 2009). The mortality rate is low, but the morbidity is high and most animals do not recover to their full production potential

(Grubman and Baxt 2004). In swine FMD primarily affects the feet, characterized by severe lesions on the coronary band, interdigital clefts and blubs, associated with severe lameness (Klein 2009). In cattle, sheep and goats, the disease is less severe, however lameness and inflammation at the cloves and mouth do occur (Alexandersen and Mowat 2005).

1.6.3. Host Response to FMDV Infection and Vaccination

FMDV triggers a rapid humoral response in either infected or vaccinated animals. Virus specific antibodies protect animals in a serotype specific manner against reinfection (Grubman and Baxt 2004; McCullough et al. 1992). The primary antigenic site recognized by neutralizing antibodies is a region in the G-H loop (residues approx. 140-160) of the VP1 protein expressed on the surface of the viral capsid (Bittle et al. 1982). In addition neutralizing antibodies target epitopes on three external structural proteins VP1, VP2 and VP3 (Grubman and Baxt 2004). 7-14 days after infection or vaccination strong protective immunity is evident (Grubman and Baxt 2004). IgM antibody appears 1st followed by IgG approximately 4-7 days post-infection. In cattle, during the early phases of infection, IgG₁ is greater than IgG₂ and IgA can be detected in the upper respiratory tract (Grubman and Baxt 2004; Mulcahy et al. 1990).

FMDV induces lymphopenia in swine and cattle (Bautista et al. 2003; Diaz-San Segundo et al. 2006; Maddur et al. 2008). Lymphopenia affects all B and T cell subsets and depletion of lymphocytes is correlated with the onset of viremia (Bautista et al. 2003; Diaz-San Segundo et al. 2006). The rapid, but transient, lymphopenia causes a reduction in the number B and T cells, adversely affecting T cell function (Golde et al. 2011).

FMDV interferes with host protein synthesis and disruption of the secretory pathway appears responsible for inhibition of new MHC class I translation and surface expression in FMDV-infected cells (Sanz-Parra et al. 1998). Specifically, a decrease in surface expression of MHC I molecules during *in vitro* FMDV infection suggests that 2B, 2C and/or 2BC may be involved in delaying the initiation of host adaptive immune response, adversely effecting the secretion of signaling molecules (Grubman et al. 2008).

Although neutralizing antibodies have been seen as the ideal immune response in FMDV infection and are the primary goal of vaccine design, they are not always correlated with protection. For example, in carrier animals, live virus can persist in the host, despite high titers of neutralizing antibodies (DiMarchi et al. 1986; Juleff et al. 2008; McCullough et al. 1992). It has been suggested that cell-mediated immunity may play an important role in clearing the virus from these persistently infected animals (Grubman and Baxt 2004). CD4⁺ and CD8⁺ T cells have been shown to induce anti-viral responses in mice, swine and cattle following infection and vaccination (Bautista et al. 2003; Collen et al. 1989; Garcia-Valcarcel et al. 1996; Grubman and Baxt 2004). For example, mice vaccinated with a DNA vaccine expressing B and T cell epitopes from FMDV structural and non-structural proteins were protected against disease development but were not able to induce an FMDV specific antibody response (Borrego et al. 2006). This was also the case when both swine and cattle were vaccinated with a replication-competent human adenovirus 5 (Ad5) vector expressing the VP1 capsid precursor; generation of virus specific neutralizing antibodies were not detected in the serum, but animals were partially protected from FMDV challenge (Sanz-Parra et al. 1999a; Sanz-

Parra et al. 1999b). More recently, Patch et al., (2011) demonstrated the induction of FMDV specific cytotoxic T cell killing by vaccination in swine and that in the absence of significant humoral immunity a killer T cell response could reduce clinical disease and decrease viremia in swine challenged with live virus (Patch et al. 2013). Therefore, combination of antibody and T cell responses to vaccination might better predict protection post challenge (Glass and Millar 1994).

Despite the prominent ability of CD8 cytotoxic T lymphocytes (CTLs) to kill virus infected cells, defining the role of CTLs in FMDV infection has gone largely unexplored in cattle, due to technical challenges in establishing assays to measure their activity. FMDV is a highly cytopathic virus, which causes infected cells to be rapidly lysed *in vitro* (Grubman and Baxt 2004). Therefore, these cells cannot be used as target cells in the CTL assay. In addition the analysis of CTL function requires an assay to measure lysis of virus infected target cells in an epitope specific, MHC restricted fashion. This is a challenge when faced with the high levels of MHC diversity in an outbred population. Only a handful of studies have demonstrated CD8+ T cell MHC class I restriction in cattle and swine in FMDV (Gerner et al. 2007; Gerner et al. 2006; Gerner et al. 2009; Guzman et al. 2010; Guzman et al. 2008; Patch et al. 2011). Despite a more dedicated focus to assess T cell immunity in FMDV, their role and importance in the disease are still unclear and should be further investigated.

1.6.4. Infection and Transmission

FMDV can spread rapidly in the form of aerosolized droplets, saliva, direct animal to animal contact or through indirect contact of personnel or contaminated

surfaces (Alexandersen and Mowat 2005). Several factors contribute to the rate of virus spread, incubation period and disease severity. These factors include viral dose, route of infection, virus strain, host species and husbandry of the animals (Quan et al. 2009). The primary route of infection is through the upper respiratory tract and following a natural infection in ruminants, initial virus replication takes place in epithelial cells (Alexandersen and Mowat 2005; Jackson et al. 2003). The dorsal surfaces of soft palate and adjacent nasopharynx are the primary sites of viral entry (Alexandersen and Mowat 2005). After the initial entry of the virus *in vivo* the virus spreads to the regional lymph nodes and into circulation (Alexandersen et al. 2001). 1-3 days prior to the onset of viremia, the virus can be detected in the oral cavity by real-time RT-PCR (Alexandersen et al. 2001).

The route of transmission depends upon the host animal. Cattle, sheep and goats can exhale up to $5.2 \log_{10} \text{TCID}_{50}$ of virus per day and pigs can produce $8.6 \log_{10} \text{TCID}_{50}$ per day, significantly contributing to the source of airborne transmission of FMDV (Alexandersen et al. 2002; Klein 2009). According to Hartnett et al., (2007) the most common route of spread of FMDV into countries free of disease is the illegal use of feeding virus-contaminated food scraps to pigs. The virus can persist in temperatures as low as -20°C and is susceptible to changes in pH post mortem in meat, but the lack of pH change in the glands and bone marrow allows for virus survival (Kitching 2005). Human infection does not seem to have a significant role in the natural infection of FMD. However, people and fomites can have a role in passive transfer of the virus from infected animals (Alexandersen and Mowat 2005).

1.6.5. FMD Virus Genome Organization

The FMD virion is a 140S particle consisting of single-stranded RNA genome and 60 copies each of four structural polypeptides, 1A, 1B, 1C, 1D (also known as VP4, VP2, VP3 and VP1, respectively), which associate to form an icosahedral shell or capsid (Carrillo et al. 2005; Jackson et al. 2003). The FMDV genome structure is comprised of an open reading frame (ORF) flanked by highly structured 5' and 3' untranslated regions (UTRs) (Carrillo et al. 2005). The capsid protects the genome whilst the virus is in transit and mediates host interactions (Carrillo et al. 2005; Jackson et al. 2003). FMDV initiates infection by receptor-mediated endocytosis, when the Arginine-Glycine-Aspartic Acid (RGD) motif within the G-H loop of VP1 binds to cellular integrin receptors ($\alpha\beta6$, $\alpha\beta3$, $\alpha\beta5$, $\alpha\beta1$) on the surface of these cells (Alexandersen and Mowat 2005; Mason et al. 1994; Monaghan et al. 2005; Rieder et al. 1994). In cell culture FMDV acquires the ability to use other receptors such as glycosaminoglycans, which do not require the RGD motif (Klein 2009). After entering the endosome, the acidic pH of this compartment causes an uncoating of the viral genome. The viral genome is then translocated across the endosomal membrane into the cytosol where it is released and serves as an mRNA template for the translation of viral proteins (Grubman and Baxt 2004). One product of translation is a polyprotein that is subsequently cleaved by a variety of virus-encoded proteases, such as leader L^{pro} and $3C^{\text{pro}}$ into mature structural and nonstructural proteins (Mason et al. 2003). Representative schematic of the FMDV genome is shown in Figure 1-6.

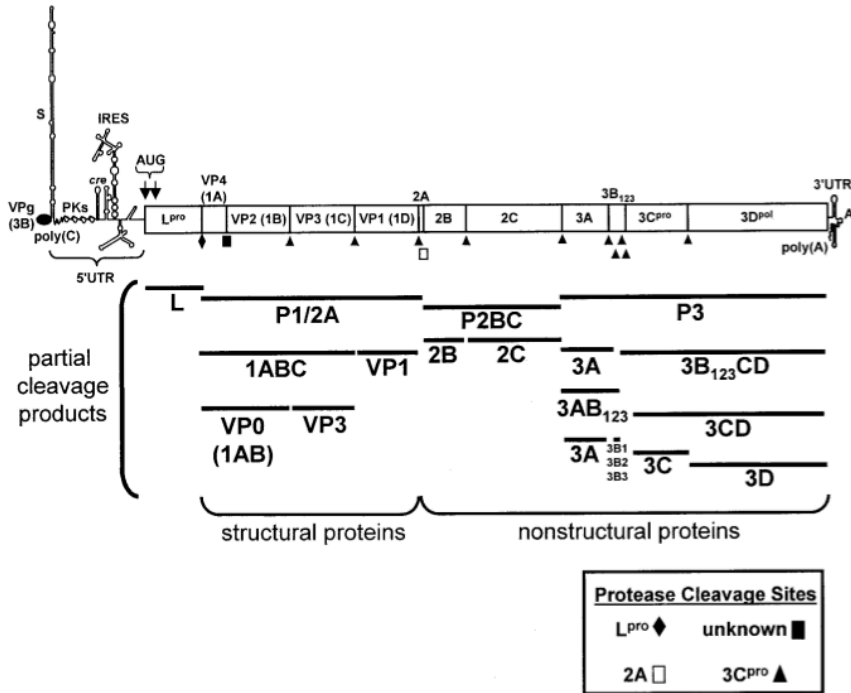


Figure 1-6: Representative map of the FMDV genome (Grubman and Baxt 2004).

1.6.6. Current Control Practices

In an effort to preserve FMD-free trade status, the primary control measure countries use is slaughter (stamping out) of infected and nearby susceptible domestic species. OIE regulations require countries that vaccinate to wait a minimum of 6 months to regain FMD-free status, as opposed to only 3 months when clearing the outbreak via stamping out (Agriculture 2015). Therefore, the prolonged loss of livestock exports and products can have devastating effects on a country's economy. One way to reduce the need for such drastic measures is with the use of improved vaccines.

Inactivated FMD vaccines have been available for more than 70 years. The use of vaccines has often been considered a last resort in response to an outbreak in disease free countries due to the trade regulations. Limitations of the current FMDV killed virus

vaccines include only partial protection and a short duration of immunity (revaccination is often required every 4 or 6 months). Virus neutralizing antibodies induced by vaccination are not protective across multiple serotypes. Additionally, in order to support DIVA (differentiating infected from vaccinated animals) in diagnostic testing for outbreak control and surveillance, some vaccine formulations need to undergo additional purification steps to remove cellular contaminants as well as some nonstructural viral proteins (Gao et al. 2012). Production of the killed virus vaccines also requires the growth of large quantities of virus, involving expensive high containment (biosafety level 3) facilities for manufacture (Doel 2005; Rodriguez and Gay 2011).

The recent development of a replication defective human adenovirus (Ad5) viral vector containing the capsid and 3C^{pro} coding regions of FMDV has addressed several of these limitations. The new vaccine has DIVA capabilities, does not require large amount of virus for manufacture and is protective in both cattle and swine (Rodriguez and Grubman 2009). It was granted a conditional license for inclusion of the vaccine in the US National Veterinary Vaccine stockpile (Grubman et al. 2010). OIE has set the goal for global eradication of FMD by the year 2030 and improved FMDV vaccines will be critical to reaching that goal and ultimately protecting US agriculture (Rodriguez and Gay 2011).

1.7. Summary

My dissertation research hypothesized that increasing our knowledge on the BoLA class I diversity in Holstein cattle and together with the translation of current technologies to assess T cell responses would lead to a better understanding of the bovine adaptive immune response to FMDV. Three ways we addressed this hypothesis was by: 1) quantify, define and develop methods to detect polymorphisms of the BoLA class I genes of Holstein cattle in a restricted population from a commercial purebred cooperative-research herd in Vermont, 2) to define the FMDV capsid protein peptide repertoire bound by BoLA class I molecules using bioinformatics and biochemical affinity and stability assays, 3) demonstrate clonal T cell expansion for specific epitope polypeptides using *ex-vivo* multi-color flow cytometric MHC-epitope complexes (tetramers), followed by IFN- γ production measured by an ELISpot assay to quantify and define the antigen specific response of Holstein cattle to FMDV vaccination.

2. CHAPTER 2: NEXT GENERATION AMPLICON SEQUENCING ACCELERATES BOLA CLASS I TYPING AND PROVIDES NEW INSIGHT TO EXPRESSED GENE DIVERSITY

This manuscript is formatted for BMC genetics methodology article.

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2.1. Abstract

Background: Major histocompatibility complex (MHC) class I molecules are polymorphic and polygenic creating challenges for standard genotyping methods typically used for other genes of interest. Current techniques for identification of expressed MHC class I alleles of cattle are time consuming and costly. Recent advances in next generation sequencing (NGS) technologies allow for the sequencing of highly polymorphic regions of the genome, such as the MHC locus. These high-throughput methodologies have been applied to class I MHC typing for several species, such as humans, nonhuman primates, and swine, but have been less commonly used in cattle.

Results: Herein, we described a comprehensive method for genotyping bovine leukocyte antigen (BoLA) class I genes from 28 Holstein cattle by using Illumina MiSeq, cloning and Sanger sequencing, and polymerase chain reaction sequence-specific primers (PCR-SSP). Illumina MiSeq was used to evaluate a 375-bp complementary DNA (cDNA) PCR amplicon spanning the highly polymorphic peptide-binding region. Cloning and Sanger sequencing was used to determine the 1kb full-length BoLA class I region. Additionally, PCR-SSP was conducted for 17 specific alleles for which primers are available. We present a streamlined bioinformatics pipeline for analyzing Illumina MiSeq data for MHC typing. The pipeline consists of rigorous requirements of sequence quality checks, consensus alignments, and *de novo* abundance based chimera detection. Using this Illumina MiSeq pipeline, we can separate true alleles from allelic artifacts, and in addition, identify potentially new alleles or variants of existing alleles. A combination of

all three different platforms, Illumina MiSeq, cloning and Sanger sequencing, PCR-SSP, will help capture the total allelic profile of an individual animal.

Conclusions: Illumina MiSeq amplicon sequencing is a higher-throughput and higher-resolution assay for efficient BoLA genotyping. While Illumina MiSeq provides a viable alternative to known methods, quality checking with stringent requirements is needed to differentiate artifacts from true alleles. This approach provides an alternative method for genotyping BoLA and improves our understanding of the genetic specificity of BoLA.

Key Words: Major histocompatibility complex; BoLA; Illumina MiSeq sequencing; genotyping; cattle

2.2. Background

The immune system plays a critical role in the maintenance of individual animals and overall herd health. The major histocompatibility complex (MHC) is a highly polymorphic region that allows the body's immune system to differentiate pathogen-infected cells from healthy cells. Expanding knowledge on which peptide epitopes bind particular MHC molecules could influence epitope discovery and vaccine design. MHC polymorphism is influenced by gene duplication, copy number variation, recombination and pathogen selection pressure [1-4]. As such, cattle MHC, also known as the bovine leukocyte antigen (BoLA), are one of the most polymorphic gene regions found in cattle [3]. In bovine, three BoLA classes exist; class I, II and III, each having a specific role in immune function. BoLA class I and II both play a role in antigen presentation, whereas the function of BoLA class III has been associated with components of the complement system [3, 5]. BoLA class II molecules present exogenous peptides to CD4 T-

lymphocytes, while BoLA class I molecules present endogenous peptides to CD8 T-lymphocytes [6]. The present study is focuses on developing methods to rapidly characterize BoLA class I diversity, an important first step in order to study the BoLA restricted immune response following infection or vaccination.

Cattle, pigs, horses and sheep, express a variable number and combination of class I MHC genes [7-9]. Cattle have six classical class I genes, 1-3 of which are typically expressed in an individual animal. There are currently 114 BoLA class I alleles, including 95 classical alleles, and 19 non-classical alleles in the Immunopolymorphism database [IPD-MHC; <http://www.ebi.ac.uk/ipd/mhc/bola/>], a database of validated MHC sequences [10]. These sequences represent the known allelic diversity of cattle. Over 40% of the BoLA sequences in IPD-MHC are from Holstein cattle. The IPD-MHC database also contains details regarding BoLA nomenclature, breed type, and serological specificity, but it is not possible to rapidly conduct searches based on these factors directly through the available interface. Additional tools are needed to better explore the information in this database, especially as the size of the database is expected to increase with the discovery of new alleles.

A variety of methods have been used to identify polymorphisms in BoLA alleles. Initially, polymorphisms of the BoLA locus were detected through use of alloantisera in complement-dependent microcytotoxicity assays [11, 12]. More recently, the use of molecular techniques such as recombinant DNA technology (cloning), chain-termination Sanger sequencing, and polymerase chain reaction using sequence specific primers (PCR-SSP) has increased the number of known BoLA alleles. The new data has changed

our understanding of BoLA molecules associated with serologically defined haplotypes, and improved the power to discriminate between BoLA molecules [4, 11, 13]. As such, improved methods for identifying existing and new BoLA alleles are needed. In cattle, the potential combination of diversity within a population and between regions is not as well characterized as humans. This leads to potential bias of BoLA alleles in the curated database. For example, the current IPD-MHC database is principally based upon European samples, which may not represent class I sequences from North American cattle, as genome wide differences have been found between the two populations [14]. Knowledge of the degree of polymorphism and expression levels of specific BoLA alleles is fundamental to understanding the functional importance of a particular molecule.

Recently, next generation sequencing technologies (NGS) has been used for high-throughput MHC genotyping in several species including humans, macaques, bison and swine [8, 15-19]. NGS technology can rapidly improve detection of rare sequences, expand information on haplotypes and allow for better understanding of inter-species variation and differences by geographic region [17, 18, 20]. To date, these techniques have not yet been widely used in cattle. The goal of the present study is to explore the utility of Illumina MiSeq (MRDNA, Shallowater, TX), as a tool for rapid BoLA genotyping. This method is capable of providing up to 500 bp read lengths, with more than 8 GB of data in less than 40 hours [21]. The Illumina MiSeq system uses the principles of sequencing by synthesis technology. This technology allows for a massively paralleled system for tracking the addition of labeled nucleotides as the DNA chain is

copied [15]. The advantages of NGS include the ability to scale up genotyping projects for larger sample sets, at a fraction of the cost, in a short period of time. Disadvantages of NGS include short read lengths, presence of indels, complications of *de novo* assembly, errors associated with GC rich sequences, and difficulty in establishing efficient procedures for distinguishing true alleles [15, 22]. Despite these limitations, NGS technology is rapidly evolving and its use in other species provides support that this technology could be applied to cattle.

Here we describe the use of Illumina MiSeq NGS technologies as a high-throughput platform for comprehensive and rapid MHC class I genotyping of Holstein cattle. Illumina MiSeq results were compared to the more traditional MHC typing methods of 1) PCR-SSP and 2) cDNA amplicon cloning and Sanger sequencing. Our results suggest that Illumina MiSeq in combination with a streamlined bioinformatics workflow will allow for rapid identification of existing and potentially new BoLA class I alleles.

2.3. Materials and Methods

2.3.1. Animals and sample collection

Whole blood samples were collected from 28 purebred Holstein steers by jugular or caudal tail venipuncture using sodium heparin vacutainer tubes (BD Bioscience, San Jose, CA) in accordance with Institutional Animal Care and Use Committee (IACUC) protocol #10-026. Steers were housed at the Miller Research Farm at the University of Vermont (UVM) Dairy Center of Excellence (DCE). Samples were transported and held

on ice until peripheral blood mononuclear cell (PBMC) isolation and mRNA extraction could be performed (within two hours of blood collection).

2.3.2. RNA Extraction and cDNA synthesis

RNA extraction and purification from isolated PBMCs was conducted using a TRIzol Reagent (Invitrogen, Grand Island, NY) and RNeasy mini kit (Qiagen, Valencia, CA), according to manufacturer's instructions, with minor modifications. Briefly, whole blood was centrifuged at 3,600 x g for 15 minutes at 4°C, for isolation of the lymphocyte monolayer. The monolayer (150 µL) was then pipetted off and added to 1 mL of TRIzol reagent and incubated for 1 minute. Ice-cold chloroform (200 µL) was added to the mixture and incubated for 1 minute and centrifuged at 1500 x g for 15 minutes at 4°C. The aqueous layer (400 µL) was combined with ice-cold ethanol (400 µL) and added to the RNeasy column. After DNase digestion manufacturer's instructions were followed. RNA samples were eluted from the column and stored at -80°C. cDNA was synthesized from these samples within a week of extraction using the Promega ImProm-II Reverse Transcription System, as per manufacturers' methods.

2.3.3. PCR-SSP

PCR-sequence specific primers (SSP) were used in accordance with previously published methods [11, 23] to amplify a total of 17 known alleles. A total of 81 steers were screened by PCR-SSP; based on these initial results, we selected 28 animals for cloning because they expressed one or more alleles of interest for parallel vaccine studies (*1*01901, 2*00801, 2*01201, 3*01701 and 4*02401*).

2.3.4. Cloning and Sanger sequencing

cDNA samples from 28 steers were used in the cloning of expressed MHC class I genes. Cloning was performed according to protocols outlined elsewhere [13] with one exception; a different set of primers was used for generating amplicons from the cDNA sample templates. The cDNA samples were amplified using full-length MHC class I sequence using “universal primers”, ILRI forward (5'-ATGRGGCCGCGARCCCT-3') and ILRI reverse (5'-TCAMACTTTAGGAACCGTGAGAG-3') (N. Svitek, personal communication). Amplified DNA was separated via a 1.5% agarose gel. The ~1,100 bp DNA band of interest was excised from the gel and purified using Qiaquick® gel purification kit (Qiagen, Valencia, CA). Purified amplicons were ligated onto a blunt-end TOPO cloning vector (Invitrogen, Grand Island, NY) following manufacturer's instructions, transformed using competent *Escherichia coli* and plated on LB Agar plates with 50µg/mL kanamycin (Teknova, Hollister, CA) to screen for competent clones using antibiotic resistance. Twenty-four positive clones for each steer were isolated and the insert was amplified using flanking primers, M13 forward and M13 reverse. Sanger sequencing was conducted on amplicons generated from up to 20 individual clones per steer, using a Big Dye Terminator v3.1 (Applied Biosystems) and cycle sequencing chemistry at the UVM cancer center DNA analysis facility. In accordance with previous studies and cost restrictions, up to 20 clones from each animal was sequenced [13]. Forward and reverse sequences were generated and aligned to create consensus sequences using Sequencher® version 5.0 (Gene Codes Corporation, Ann Arbor, MI). Consensus sequences were subsequently imported into GeneiousPro® version 6.1.8

(Biomatters, Auckland, NZ) and compared to known alleles using NCBI basic local alignment search tool (BLAST) and the IPD-MHC database for confirmed sequences [<http://www.ebi.ac.uk/ipd/mhc/bola/>].

2.3.5. Illumina MiSeq library generation and sequencing

Samples for Illumina MiSeq sequencing were generated from a 410 bp amplicon of the hyper-variable region spanning exons two and three (α -1 and 2) of the class I gene using “universal primers” bov 7 and bov 11 [4]. Samples were shipped overnight to molecular research DNA laboratories (MRDNA, Shallowater, TX), for Illumina MiSeq using a 500 cycle MiSeq reagent kit version 2.0. Unique DNA barcodes were annealed to amplicon DNA from each sample to allow for sample source identification throughout the process. Samples from 28 animals were multiplexed into a single sequencing run, divided into two lanes on the flow cell. Multiplexed samples were purified using calibrated Ampure XP beads (New England Biolabs, Ipswich, MA). The purified, barcoded samples were used to prepare a DNA library, following Illumina TruSeq DNA library preparation protocol (MRDNA, Shallowater, TX). Sequence data was processed by binning individual barcodes for each sample (steer number), and aligning amplified sequences to all known sequences from the IPD-MHC database. During this process several steps were taken to ensure accuracy and to develop a streamlined bioinformatics pipeline: 1) Paired end sequences were de-multiplexed; 2) only those reads with an average Phred score of Q25 [24] were included, 3) a perfect overlap between forward and reverse reads was required to create a consensus sequence; 4) merged reads needed to be exactly 418 bp in length, consisting of the barcode (8 bp), forward and reverse primers

(35 bp), and bov 7 and bov 11 region (375 bp). These merged sequences were trimmed to just the amplicon region (375bp) and then grouped and counted by using Bioawk [25] (<https://github.com/lh3/bioawk>). Only those reads with a minimum threshold of 100 reads per sample were included; 5) the resulting clusters were checked for chimeras using VSEARCH version 1.0.16 (<https://github.com/torognes/vsearch>, [10.5281/zenodo.15524](https://doi.org/10.5281/zenodo.15524)) *de novo* assembly, which uses abundance as a reference. VSEARCH default parameters were used, with the exceptions of adjusting the minimum score to 1.2 and minimum divergence score to 1.5 for chimera detection. Based on this criteria, any sequence classified as a putative new chimeric sequence were removed from further analysis; 6) Non-chimeric sequences were aligned to the reference file containing BoLA sequences from IPD-MHC; 7) Putative new alleles that were not 100% match to the reference database were compared across sequences from the steers. Sequences were only considered as “new” if the sequence occurred in three or more steers.

2.3.6. Ruby script and Shannon diversity plot

A Ruby script was developed to access and manipulate data contained within the bovine IPD-MHC (Available at https://github.com/MitalGitHub/IPD_MHC and <http://asci.uvm.edu/labs/barlow/tools.html>). The script allows a search through the BoLA class I alleles in IPD by allele name, serological specificity, or breed. The script utilizes Ruby gems, such as Bioruby [26], to obtain the nucleotide or amino acid FASTA files associated with the search. Sequences downloaded from IPD-MHC were compiled for the MiSeq reference file. A Shannon diversity plot was generated using sequences downloaded from IPD-MHC and Protein Variability Database server [27].

2.3.7. Data analysis

Analysis of sequences and primer site variation was performed using Geneious 6.1.8 (Biomatters, Auckland, NZ). Data generated from all platforms were gathered, stored and manipulated using Excel (Microsoft). All scripts used in this study are available at https://github.com/MitalGitHub/IPD_MHC. Analysis and comparison of all three methods was analyzed in Excel.

2.4. Results

Three techniques were used to assess MHC class I sequence diversity in 28 Holstein steers: 1) PCR using sequence specific primers, 2) cDNA amplicon cloning followed by Sanger sequencing and 3) Illumina MiSeq amplicon sequencing.

2.4.1. PCR-SSP

Fifteen of the 17 alleles were detected by PCR-SSP (Table 2-1). *6*01301* and *6*04101* were not found in any of the steers by PCR-SSP. The number of alleles found positive by PCR-SSP varied between 2-7 for a given animal.

2.4.2. Cloning and Sanger sequencing

We obtained full-length sequence from 650 clones obtained from the 28 steers. The most common allele found by cloning and Sanger sequencing was *1*01901*, present in 78% (22/28) of the animals (Table 2-1). The other alleles of interest for the parallel peptide binding studies were found less frequently by this method: *2*00801* (18%), *2*01201* (0%), *3*01701* (22%), and *4*02401* (29%) (Table 2-1). The low frequency of

these alleles by cloning and Sanger sequencing led to investigation of the ILRI primer binding sites, primarily to identify if poor binding here could be the reason for decreased numbers of alleles found (Table 2-2). The 30 alleles listed in Table 2-2 were chosen for exploration because they were found by either PCR-SSP or Illumina MiSeq. For example, a sample with a positive result by PCR-SSP along with high number of ILRI primer mismatches and negative result by cloning indicates that there is the possibility of false negatives due to preferential selection using this method. While it does not account for every instance of discordance between PCR-SSP and cloning results, or Illumina and cloning results, there appears to be a strong trend between the number of mismatches in the ILRI forward primer and frequency by cloning. One example of this was shown with 2*01201, there are a total of six mismatches in the ILRI forward primer with this allele (Table 2-2). This could be a possible explanation as to why this allele was not found by cloning. There were no significant differences (more than one mismatch) between allelic and primer sequences in the ILRI reverse region, so this is not likely to be an issue (data not shown). Another example of discordance between Illumina MiSeq and cloning is shown with 2*00801. This allele was found 23 times by Illumina MiSeq, but was found only five times by cloning. Again, this allele has a relatively high number of mismatches in the ILRI forward primer, which could explain why it was not found.

2.4.3. Illumina MiSeq

Illumina MiSeq version 2.0 sequencing was performed on amplicons generated by bov 7 and bov 11 PCR. Bov 7 and bov 11 primers were chosen as they bind highly conserved sites, flanking the most highly polymorphic region within the MHC class I,

and were used to generate universal α -1 and 2 amplicons from cDNA templates for NGS (Figure 2-1). The average number of reads per sample from Illumina MiSeq was 33,844. Further analysis of bov 7 and bov 11 binding regions was performed *in silico*, and revealed a high number of polymorphisms in these regions (data not shown). MHC class I sequences were downloaded from the cattle IPD-MHC database using a Ruby script, and a reference file for read alignments was created. Several alleles are identical in the α -1 and 2 regions, therefore, these were combined under the same name in the reference file (Table 2-3).

2.4.4. Comparison of platforms

The Illumina MiSeq results for the 28 animals that were tested across all platforms were compiled and compared to results from both PCR-SSP and cloning and Sanger sequencing (Table 2-4). A total of 450 sequence reads conformed to our initial quality criteria. However, after passing the sequences thru the VSEARCH program, 181 of those sequences were classified as chimeric and removed from further analysis. The median percentage of chimeric reads per sample (steer) was 29%. As a result 269 individual sequences passed all stringent requirements set in our pipeline. Using these criteria, a range of 1 to 11 alleles per sample were found as a perfect match to a previously known allele. The number of positive reads per sample meeting the quality criteria varied greatly, from 115 to 100,952 (Table 2-4). This suggests there may not be a relationship between the number of positive reads and success of allele calling, as even those samples with a lower total read number had 100% matches to known alleles. This is important to consider when developing a bioinformatics pipeline, as discarding samples

with a low number of reads may lead to false negatives. The summary of the alleles for each sample is shown in Figure 2-2. All 28 steers had at least one of the target alleles: *1*01901*, *2*01201*, *2*00801*, *3*01701*, and *4*02401*. However, *1*01901* was only found by Illumina MiSeq in one steer (steer 182) (Figure 2-2). As demonstrated in Figure 2-2, it appears some alleles are found at a higher proportion of reads as compared to others. For example, *2*00801* was found with an average of 7,469 reads as compared to *2*01801* was found with an average of 2,555 reads. Illumina MiSeq was able to capture some alleles that were missed by PCR-SSP and cloning. For example, for steer 176 we found *2*01601* by Illumina MiSeq, but when screened by PCR-SSP and cloning this allele was not found (Table 2-1 and 2-4). Another example of this is shown in steer 65, Illumina MiSeq found *3*00201*, but this allele was not found by the other two methods. Additionally, Illumina MiSeq was able to find four alleles: *3*03601*, *3*03701*, *NC1*00501* and *NC1*00201* that were not found by cloning and for which PCR-SSP primer pairs are not currently available. However, seven alleles: *1*02001*, *1*02101*, *3*01702*, *6*01401*, *6*01501*, *NC1*00301* and *NC3*00101* were not found by Illumina MiSeq, but were found by cloning or PCR-SSP (Table 2-1). We applied the stringent requirements from our bioinformatics pipeline to classify potentially new alleles. In order to be classified as a potentially new allele, the exact sequence had to meet all quality filters, chimera checkpoints and had to be found in at least three different animals. Based on these criteria Illumina MiSeq was able to identify 41 potentially new alleles or allelic variants based on the α -1 and 2 regions we sequenced. Since only full-length sequence is

accepted to IPD-MHC, in order to characterize a new alleles or variants additional work needs to be done to validate these sequences.

2.5. Discussion

Efficient and reliable genotyping is a prerequisite for understanding the role of MHC in the immune response after vaccination or infection. Successful BoLA genotyping of large herds depends on robust amplification, high quality sequence reads, and rapid analysis workflows. There are several challenges that are presented by the current MHC class I typing methods compared in this paper. First, allelic identification by PCR-SSP is limited by the available number of PCR-SSP primers, and new primer sets need to be designed for any additional alleles of interest. In the IPD-MHC database there are over 100 BoLA class I alleles, and this number is likely to grow over time as new alleles and variations are discovered. Development of sequence specific primers for every allele currently in the database and each subsequent new allele does not seem feasible. Second, PCR-SSP does not allow for the discovery of new alleles or variants. Third, PCR-SSP testing of individual alleles using separate primer sets is low-throughput. Therefore, an alternative method for MHC typing cattle is the use of molecular cloning. This method allows for the amplification of full-length MHC sequences (approximately 1kb), using “universal primers”. Cloning is generally considered advantageous compared to PCR-SSP, since the full-length sequences allow for a more complete representation of allelic diversity within a herd, as one is not constrained to the specific primers chosen for allelic testing. However, cloning is quite costly and a labor intensive process and was limited by the number of clones sequenced as shown in this study and others [13, 22].

Another potential flaw of this methodology is the use of “universal primers”, which can lead to amplification bias causing certain alleles to be missed (Table 2-2). Currently, for submission of a new BoLA allele to IPD, only full-length sequence determined by cloning and Sanger sequencing is accepted. However, Illumina MiSeq has the potential to identify additional new sequences, but these sequences would need to be further validated by cloning and Sanger sequencing at this time. There are merits to each typing system, but in a study such as this where the goal is to quickly assess the allelic background of the herd as well as build upon existing data, previous methodologies are lacking. Neither of the two existing platforms, PCR-SSP nor molecular cloning, present researchers with a true “gold standard” for comparison and as such are lacking as diagnostic tools.

Recently, NGS has been used for MHC genotyping for a number of species such as humans, macaques, bison, guppy and swine [8, 15-19, 28]. For the characterization of BoLA class I variation in our Holstein cattle population we used boV 7 and boV 11 primers, which were previously designed to amplify the majority of BoLA class I sequences spanning exons 2 and 3 [4, 29, 30]. These primers were also applied in 454 pyrosequencing to genotype the variation in European bison [19]. Exon 2 and 3 encode for the highly polymorphic α -1 and 2 domains composing the peptide-binding groove of the MHC class I molecule. This region is not only the most variable and important in antigen presentation, but allows for 92% discrimination among the known BoLA alleles. Therefore, we focused our efforts specifically targeting this region to determine the allelic diversity in cattle using the Illumina-MiSeq system. We validated the NGS approach of Illumina MiSeq for MHC class I typing in cattle by comparing the results

generated to previous typing methods. This experiment has laid the foundation for establishing a NGS MHC genotyping protocol in cattle; however, there are several limitations that must be addressed before it can be used as a “stand alone” platform for large-scale herd or population level MHC typing. Such limitations include: determining the number of polymorphisms or single nucleotide polymorphisms (SNPs), distinguishing chimeras from true alleles, and creation of an absolute read threshold for identifying expressed genes. In addition to addressing the feasibility of Illumina MiSeq, several issues within each of the typing platforms were discovered and explored.

Cloning and sequencing expressed genes has limitations for large-scale population genotyping. Possible preferential amplification of alleles, mismatches between primer and target sequences, and cost are all issues associated with this platform. A major limiting factor is the number of clones that can be sequenced in a single run. In this study the goal was to sequence at least 20 clones per animal, as this number could be readily extracted in a single run and fit on one sequencing plate to limit costs. However, this practice may affect the probability that all expressed alleles are present among the clones selected for sequencing. If we consider only sequencing results from clones in this study, our results appeared to conflict with previous published allele combinations in known haplotypes [1, 3]. For example, the alleles *I*01901* and *2*00801* are reported to be co-expressed in all animals of the common haplotype A12, which was confirmed by PCR-SSP typing for each individual steer enrolled in this study. However, by cloning the allele *2*00801* was only found in five steers; whereas *I*01901* was identified in 22 animals. As previously discussed, many of the alleles presumed to be in our population have at least

one mismatch to the forward cloning primer, and for *2*00801* there are five mismatches. In addition to sheer number of primer mismatches, many of these occur at the 3' end of the primer sequence, which would likely impair annealing of the primer sequence and subsequent amplification of those alleles. We suspect this leads to the preferential amplification of those alleles with either perfect matches or a low number of mismatches to the primer, and biased our cloning results in favor of those molecules. Additional research is needed to identify alternative primers that might be used for cloning full-length sequences. For example, Birch et al. previously reported using a combination of primer pairs for cloning full-length expressed sequences in British Friesian and Holstein cattle [4]. However, our prior experiments with these primers were unrewarding (M. Pandya, unpublished).

Similarly, several alleles were not found by Illumina MiSeq (Table 1). Using the same haplotype example, A12, *2*00801* was found in every sample it was predicted to appear in, but *1*01901* was only found in one steer by Illumina. Mismatches in the bov 7 and bov 11 primer sequences could lead to decreased binding affinity during the PCR methods. It is unknown as to what may be missed by these reactions due to poor specificity of these “universal primers” and presents an issue faced by any amplification process.

Additionally there were nine alleles that shared overlapping sequence identity in the peptide binding groove of the MHC molecule (Table 2-3). We presume these alleles are similar in function, however this is yet to be determined. In order to distinguish

between these alleles, additional targeted PCR of the full length BoLA region would need to be performed.

Other potential issues with Illumina MiSeq amplicon sequencing have been reported. Several sources have noted the presence of chimeric sequences in high-throughput sequencing data [22, 31]. Chimeras can be generated during PCR amplification when there is incomplete extension of a sequence, which then anneals to another “parent” sequence during the next round of the reaction. Chimeras can also be generated during the *de novo* assembly process. In order to address these issues, the VSEARCH program was used to detect putative chimera sequences, however the parameters need to be adjusted to identify these sequences correctly. For example, alleles 3*01701 and 3*00201 were initially classified as chimeric sequences by the VSEARCH program, even though the reads were a 100% match to the reference alleles. This highlights the need for an optimized bioinformatics pipeline for BoLA molecules specifically, as following the default parameters would result in loss of data. In this case, we were able to manually classify these sequences as non-chimeric because they could be verified by other typing methods. Another potential solution of dealing with chimeric sequences and potential artifacts is to optimize the PCR cycles during the library preparation steps [32].

One potential concern that arose after analysis of the Illumina MiSeq data is that we observed some steers had more alleles (up to 11) than previously reported in the literature. In our streamlined bioinformatics pipeline, we required reads to have a minimum threshold of 100 reads per sample. Based on these criteria, for example, steer

182 has 11 alleles (Table 2-4) identified by Illumina MiSeq. However, many of these alleles had relatively low sequence reads. Increasing the read threshold to 200 reads would reduce the number of predicted alleles in a sample, but it is not clear whether by shifting the read threshold we would be eliminating sequencing artifacts or true expressed sequences. For example, in steer 182 both 1*01901 and 1*03101 were found at <200 reads per sequence, yet 1*01901 was found by all 3 typing methods in this steer, and 1*03101 was only found by MiSeq amplicon sequencing. However, following the assumption that artifacts should be rarer among individual's reads than most true alleles, increasing the threshold will indeed result in less artifacts [33], although in some cases possibly at the expense of eliminating true alleles. Additional experiments are required to resolve some of these challenges if NGS is to be applied to BoLA typing.

In addition to the challenges faced with NGS Illumina platform, important issues with the two established methods were also raised. Characterization of primer polymorphisms and preferential selection allow past and present results to be viewed with a more critical lens. The problem with every system is the challenge of optimizing high sensitivity and specificity in each assay. In PCR-SSP we lack primers for every known allele and therefore cannot test all 114 known BoLA class I alleles or discover potential new alleles or variants. In cloning and Illumina the primers that we select influence which alleles get amplified (Table 2-2). Another challenge in the Illumina system is determining where to set the appropriate read threshold cutoff in order to reduce the number of false positives. Seven alleles were found by the other 2 methods, but not by Illumina MiSeq (Table 2-1). NC1*00301 was not specifically found by Illumina MiSeq

because it was identical in the amplicon region to NC1*00201 (Table 2-3). Two additional alleles (6*01301 and 6*04101) were not found by any genotyping method we used.

Despite these limitations, Illumina MiSeq allowed for identification of more alleles compared to PCR-SSP protocols and is not limited by the number of clones produced, all in a single run. The Illumina MiSeq system had far fewer “misses” than the other two platforms and provides the best overall coverage of our population (Table 2-1). There is far less time spent on sample preparation, far fewer resources consumed, and a much faster analysis time once the initial bioinformatics workflow is established. Per-sample cost for Illumina does vary depending on the level of multiplexing and other factors, such as sample and library preparations, but on average is estimated at ~\$75-\$100 per each sample. Illumina has higher throughput and higher resolution than PCR-SSP, cloning and Sanger sequencing. It is possible that the most comprehensive account of genetic expression in a population could be gathered by a combination of methods, rather than a single method. For example, our data suggests combining Illumina sequencing and PCR-SSP results is a tractable alternative. When used in parallel the two methods appear to provide a complete picture in a relatively short time frame, as initial high-throughput results could guide PCR-SSP testing, limiting the number of individual reactions that would need to be performed. Utilizing the strengths of different high-throughput sequencing platforms is another option, as a combination of two different NGS platforms (Roche 454 and Illumina MiSeq) were used to discover novel full-length MHC class I alleles in rhesus macaques [34].

2.6. Conclusions

The recent development of NGS technologies have rapidly increased knowledge on human MHC alleles while in bovine, the use of NGS for MHC genotyping is just being developed. Despite some challenges, the present study demonstrated Illumina MiSeq maybe an efficient high throughput genotyping platform for amplicon sequencing of complex multilocus systems such as bovine MHC. This study demonstrated that Illumina MiSeq provides a robust, cost effective, alternative to known methods, however, stringent quality checking is required. The ability to rapidly and comprehensively genotype BoLA within a herd will allow for identification of allele specific BoLA peptide binding motifs. Therefore, this will provide to a better understanding of the BoLA restricted immune response by studying peptide-BoLA interactions and ultimately improve epitope based vaccine design.

2.7. Abbreviations

BLAST: Basic local alignment search tool

BoLA: Bovine leukocyte antigen

cDNA: Complementary DNA

DCE: Dairy center of excellence

EDTA: Ethylenediaminetetraacetic acid

HLA: Human Leukocyte Antigen

IPD-MHC: Immunopolymorphism database

MHC: Major histocompatibility complex

MRDNA: Molecular research DNA laboratories

NGS: Next generation sequencing

PBMC: Peripheral blood mononuclear cells

PCR-SSP: Polymerase chain reaction sequence-specific primers

RFLP: Restriction fragment length polymorphisms

USDA: United States Department of Agriculture

UVM: University of Vermont

2.8. Competing interests

The authors declare they have no competing interests.

2.9. Authors' contributions

MP, KE and JB designed the study. MP performed the extraction of mRNA, prepared cDNA, performed experiments, analyzed the cloning data, generated bioinformatics analysis and drafted the manuscript. KE contributed to experimental laboratory work, analysis of NGS data, and writing the manuscript. JV contributed to development of the bioinformatics pipeline, VSEARCH analysis and alignment of NGS sequence reads. JB advised on data analysis, and JB and WG participated in coordination and design of the study, and contributed to final modifications of the manuscript. All authors read and approved the final manuscript.

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2.12. Figures and tables

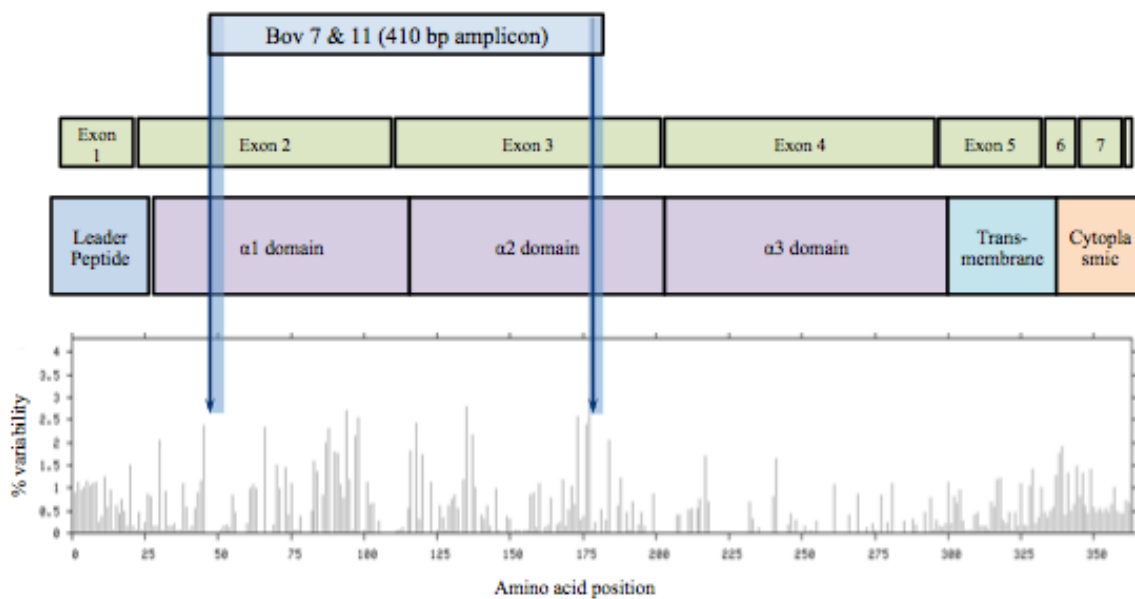


Figure 2-1: Shannon Diversity Plot of the BoLA gene aligned to linear representations of the gene. The 410 base-pair (bp) amplicon generated by the bov 7 and bov 11 primers for Illumina MiSeq is illustrated, with light shaded bars representing the primer binding regions of low sequence diversity.

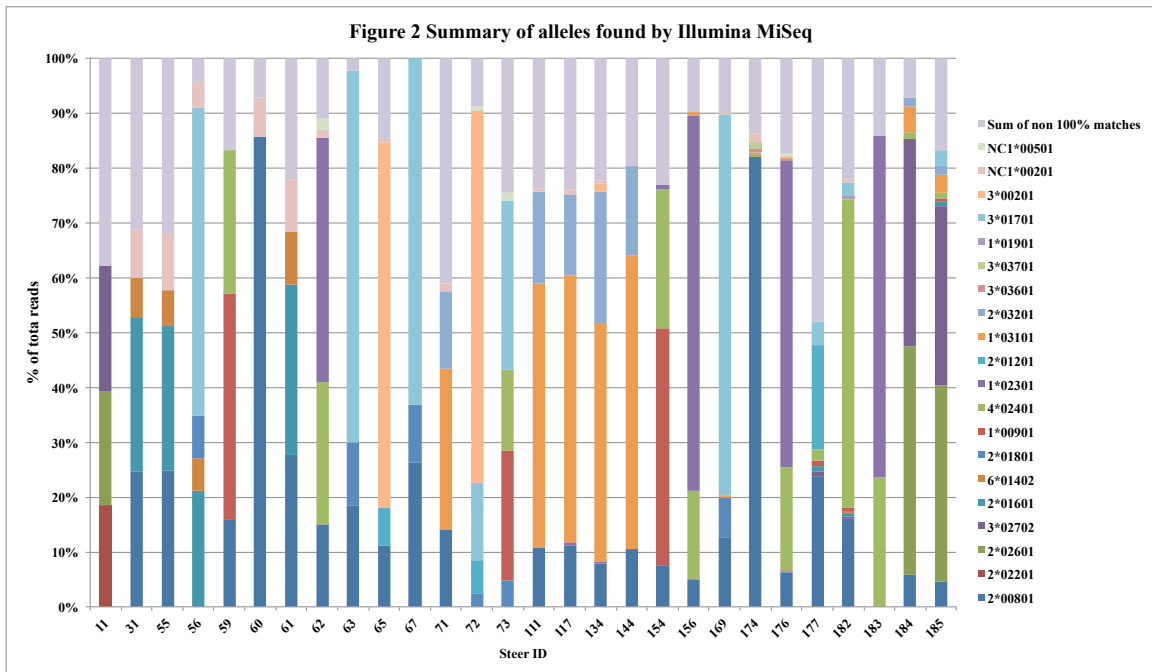


Figure 2-2: A comparison of MHC class I allele sequences across 28 steers generated from Illumina MiSeq. Reads that were <100% match to alleles in the reference file were summed and classified as “not 100% match” (shown in gray). Individual alleles displayed as single colors were 100% match to alleles in IPD-MHC database.

Table 2-1: Summary of allele coverage by each platform in 28 steers

Steer ID	1*00901	1*00911	1*02101	1*02501	1*03101	2*00801	2*01201	2*01601	2*01801	2*02201	2*02601	2*03201A	3*00201	3*01701	3*01702	3*02702	3*03601	3*05701	4*02401	6*01301	6*01302	6*01401	6*01402	6*01501	6*04101	NC1*00201	NC1*00301	NC1*00601	NC3*00101				
11			P																														
31	PC			A		PI	P															P	CI									C	
55	PC			PI																		PC	CI									C	
56						A	PI															P	CI									C	
59	I		P			A	P																									C	
60	PC			A																												C	
61	PC			A		PI																										C	
62	P			PI																												C	
63	PC	P				PI																										C	
65	PC					PI																											C
67	PC					PI																											C
71	PC	P				PI	P																									C	
72						PI																											C
73	CI						P																										C
111																																	C
117		PC	PC			CI																											C
134		PC	PC			CI																											C
144		PC	P			CI	PI	P																									C
154	CI	PC	C			PI																											C
156		PC	C			A																											C
169		PC	C			I	PI																										C
174		PC	C			I	PI	P																									C
176	I	PC	C			A																											C
177	I	PC				PI																											C
182	I	A	P			I	PI																										C
183						A																											C
184		PC	PC			I	PI	P																									C
185	I	PC	PC			I	PI																										C

Typing methods are abbreviated: PCR-SSP (P), Cloning and Sanger sequencing (C), Illumina MiSeq v2.0 (I), all three methods (A)
 *BovA alleles with PCR-SSP primers available

Table 2-2: ILRI forward primer sequence variations

Allele	Forward primer sequence 5' → 3'												# of variations	# of animals the allele was found in	Total # clones found for this allele			
	A	T	G	G	C	C	G	C	G	A	R	C				C	C	T
1*00901																0	22	4
1*01901																0	10	34
1*02001																0	0	0
1*02101																0	3	11
1*02301																5	3	15
1*03101																5	0	1
2*00801																6	0	0
2*01201																6	1	2
2*01601																6	0	0
2*01801																6	1	1
2*02201																6	0	0
2*02601																6	0	0
2*03201N																6	4	10
3*00201																4	1	14
3*01701																4	6	37
3*01702																0	6	11
3*02702																5	3	29
3*03601																0	0	0
3*03701																4	0	0
4*02401																4	0	77
6*01301																0	8	0
6*01302																1	0	0
6*01401																5	0	0
6*01402																5	2	5
6*01501																6	4	23
6*04101																5	0	0
NCI*00201																0	0	0
NCI*00301																4	0	0
NCI*00501																4	2	2
NC3*00101																4	0	0
																4	17	33

Table 2-3: Alleles containing identical sequence in $\alpha 1$ - $\alpha 2$ region, combined in the reference file

Table 3 Alleles containing identical sequence in $\alpha 1$ - $\alpha 2$ region, combined in the reference file

2*00601	1*00602	=	2*00601
2*01601	2*01602	=	2*01601
2*01801	2*01802	=	2*01801
2*02601	2*02603	=	2*02601
2*00801	2*00802	=	2*00801
3*00101	3*00102	3*00103	= 3*00101
3*00402	3*05301	=	3*00402
NC1*00201	NC1*00301	=	NC1*00201

Table 2-4: Illumina MiSeq results compared across platforms

Table 4 Illumina MiSeq results compared across platforms

Steer ID	BoLA allele	Read counts	PCR (Y/N)	Cloning (Y/N)
11	2*02201	14176	Y	Y
	2*02601	16026	N/A	N
	3*02702	17753	N/A	Y
31	2*00801	5740	Y	Y
	2*01601	6593	Y	N
	6*01402	1659	Y	Y
	NC1*00201	2072	N/A	N
55	2*00801	8189	Y	N
	2*01601	8717	Y	N
	6*01402	2175	Y	Y
	NC1*00201	3493	N/A	N
56	2*01601	3346	Y	Y
	2*01801	1214	Y	N
	3*01701	8849	Y	Y
	6*01402	932	Y	Y
	NC1*00201	730	N/A	N
59	1*00901	6208	N/A	N
	2*00801	2389	Y	Y
	4*02401	3934	Y	Y
60	2*00801	15690	Y	Y
	NC1*00201	1289	N/A	N
61	2*00801	5252	Y	N
	2*01601	5915	Y	Y
	6*01402	1818	Y	Y
	NC1*00201	1814	N/A	N
62	1*02301	13346	Y	N
	2*00801	4525	Y	N
	4*02401	7765	Y	Y
	NC1*00201	424	N/A	N
	NC1*00501	607	N/A	N
63	2*00801	4704	Y	N
	2*01801	2933	Y	N
	3*01701	17249	Y	Y
65	2*00801	8987	Y	N
	2*01201	5799	Y	N
	3*00201	54155	N	N

	NC1*00201	460	N/A	N
67	2*00801	3469	Y	N
	2*01801	1389	Y	N
	3*01701	8330	Y	Y
71	1*03101	21159	N/A	N
	2*00801	9992	Y	N
	2*03201N	9996	N/A	N
	NC1*00201	1052	N/A	N
72	2*01201	3199	Y	N
	2*01801	1306	Y	N
	3*00201	36240	N	Y
	3*01701	7586	Y	Y
	NC1*00501	460	N/A	N
73	1*00901	11683	N/A	Y
	2*01801	2373	Y	N
	3*01701	15291	Y	Y
	4*02401	7248	Y	Y
	NC1*00501	627	N/A	N
111	1*03101	16617	N/A	Y
	2*00801	3715	Y	N
	2*03201N	5811	N/A	Y
	NC1*00201	186	N/A	N
117	1*02301	191	N	N
	1*03101	15240	N/A	N
	2*00801	3522	Y	N
	2*03201N	4596	N/A	Y
	NC1*00201	284	N/A	N
134	1*02301	189	N	N
	1*03101	20602	N/A	Y
	2*00801	3710	Y	N
	2*03201N	11428	N/A	Y
	3*00201	694	N	N
	NC1*00201	247	N/A	N
144	1*03101	10147	N/A	N
	2*00801	1996	Y	N
	2*03201N	3078	N/A	Y
154	1*00901	11091	N/A	Y
	1*02301	216	Y	N
	2*00801	1940	Y	Y
	4*02401	6531	Y	Y

156	1*02301	19811	Y	Y
	1*03101	153	N/A	N
	2*00801	1473	Y	N
	4*02401	4656	Y	Y
169	1*03101	280	N/A	N
	2*00801	10374	Y	N
	2*01801	6115	Y	N
	3*00201	956	N	N
	3*01701	57220	Y	Y
	NC1*00201	237	N/A	N
174	1*02301	171	N	N
	1*03101	303	N/A	N
	2*00801	59252	Y	N
	2*03201N	177	N/A	N
	3*00201	1045	N	N
	3*03601	237	N/A	N
	3*03701	944	N/A	N
	4*02401	175	N	N
	NC1*00201	1236	N/A	N
176	1*00901	194	N/A	N
	1*02301	45383	Y	Y
	1*03101	428	N/A	N
	2*00801	5064	Y	N
	2*01601	189	N	N
	3*00201	1017	N	N
	4*02401	15160	Y	Y
	NC1*00201	359	N/A	Y
	NC1*00501	318	N/A	N
177	1*00901	166	N/A	N
	2*00801	3948	Y	N
	2*01201	3178	Y	N
	2*01601	172	N	N
	3*00201	100952	N	Y
	3*01701	685	N	N
	3*02702	149	N/A	N
	4*02401	345	N	N
182	1*00901	258	N/A	N
	1*01901	138	Y	Y
	1*03101	121	N/A	N
	2*00801	5597	Y	N

	2*01601	226	N	N
	3*00201	644	N	N
	3*01701	846	N	N
	3*02702	151	N/A	N
	4*02401	19615	Y	Y
	6*01402	117	N/A	N
	NC1*00201	268	N/A	N
183	1*02301	13759	Y	Y
	4*02401	5205	N	Y
184	1*03101	412	N/A	N
	2*00801	521	Y	N
	2*02601	3744	N/A	N
	2*03201N	153	N/A	N
	3*02702	3408	N/A	Y
	4*02401	115	N	N
185	1*00901	140	N/A	N
	1*03101	708	N/A	N
	2*00801	1019	Y	N
	2*01601	179	N	N
	2*02601	8028	N/A	Y
	2*03201N	359	N/A	N
	3*01701	650	N	N
	3*02702	7360	N/A	Y
	4*02401	256	N	N

*Results are listed as “N/A” where no sequence specific primer was available

**3. CHAPTER 3: A MODERN APPROACH FOR EPITOPE PREDICTION:
IDENTIFICATION OF FOOT-AND-MOUTH-DISEASE VIRUS
PEPTIDES BINDING BOVINE LEUKOCYTE ANTIGEN (BOLA) CLASS
I MOLECULES**

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3.1. Abstract

Major histocompatibility complex (MHC) class I molecules regulate adaptive immune responses through the presentation of antigenic peptides to CD8⁺ T-cells. Polymorphisms in the peptide binding region of class I molecules determine peptide binding affinity and stability during antigen presentation, and different antigen peptide motifs are associated with specific genetic sequences of class I molecules. Understanding bovine leukocyte antigen (BoLA), peptide-MHC class I binding specificities may facilitate development of vaccines or reagents for quantifying the adaptive immune response to intracellular pathogens, such as foot and mouth disease virus (FMDV). Six synthetic BoLA class I (BoLA-I) molecules were produced and the peptide binding motif was generated for five of the six molecules using a combined approach of positional scanning combinatorial peptide libraries (PSCPL) and neural network based predictions (*NetMHCpan*). The updated *NetMHCpan* server was used to predict BoLA-I binding peptides within the P1 structural polyprotein sequence of FMDV (strain A24 Cruzeiro) for BoLA-1*01901, -2*00801, -2*01201 and -4*02401. Peptide binding affinity and stability was determined for these BoLA-I molecules using the luminescent oxygen channeling immunoassay (LOCI) and scintillation proximity assay (SPA). The functional diversity of known BoLA alleles was predicted using the *MHCcluster* tool and functional predictions for peptide motifs were compared to observed data from this and prior studies. Results of these analyses showed that BoLA alleles cluster into three distinct groups with the potential to define 'BoLA supertypes'. This streamlined approach identifies potential T-cell epitopes from pathogens, such as FMDV and provides insight

into T-cell immunity following infection or vaccination.

Keywords: MHC class I, motif, peptide, FMDV, epitope, Immunoinformatics

3.2. Introduction

Peptide binding to major histocompatibility complex (MHC) class I molecules is the most selective step in determining immunogenicity and immunodominance of antigens from intracellular pathogens such as viruses (Yewdell and Bennink 1999). The alpha-one and alpha-two domains of MHC class I molecules form a binding cleft that presents short poly-peptides derived from cytosolic and nuclear proteins. Given the thousands of potential 8 to 11 amino acid poly-peptides that can be generated from the proteins of intracellular pathogens, antigenic peptides that stably bind to major histocompatibility complex (MHC) class I molecules are relatively rare. The peptides that bind to class I molecules often share common features (i.e., motifs); this is a function of key amino acid residues in the binding pockets of the MHC molecules. The antigenic peptides (epitopes) presented in the context of MHC class I molecules on the surface of virus-infected cells or activated antigen presenting dendritic cells are recognized by T-cell receptors (TCRs) on CD8⁺ T lymphocytes. Upon recognition of the peptide-MHC class I complexes naïve CD8⁺ T-cells expand and differentiate into cytotoxic T lymphocytes (CTLs). These cells recognize and kill virus-infected cells through several mechanisms such as perforin, granzyme, and Fas ligand pathways (Groscurth and Filgueira 1998).

MHC class I molecules can be characterized based on their ability to bind specific peptide motifs (Stryhn et al. 1996). MHC class I molecules preferentially bind peptides of

8 to 11 amino acids, most commonly with dominant anchor residues located at positions 2 and 3 relative to the amino terminus, and at the C-terminus. Residues at other positions (e.g., positions 1, 4, 5, 6, 7, 8) may also influence peptide binding and affect TCR binding to the MHC-peptide complex (Calis et al. 2013; Frankild et al. 2008). For example, a large fraction of human leukocyte antigen (HLA) alleles are associated with specificities for proline in position two (P2) and hydrophobic residues at the C-terminus (B7-supertype), or with hydrophobic residues in P2 and positive charges at the C-terminus (A3-supertype) (Sidney et al. 2008). Similar anchor residues are reported in other species such as cattle, pigs, birds, and chimpanzees (Follin et al. 2013; Hansen et al. 2014; Pedersen et al. 2012; Thomsen et al. 2013). Among cattle, there are currently 95 classical MHC class I alleles listed in the Immuno-Polymorphism Database (IPD-MHC; <http://www.ebi.ac.uk/ipd/>) although additional alleles have been reported (Hansen et al. 2014). Peptide binding motifs or epitopes have been described for at least 13 bovine leukocyte antigen class I (BoLA-I) molecules (Bamford et al. 1995; De Groot et al. 2003; Gaddum et al. 1996a; Gaddum et al. 1996b; Graham et al. 2008; Guzman et al. 2010; Hansen et al. 2014; Hart et al. 2011; Hegde et al. 1999; Hegde et al. 1995; Hegde and Srikumaran 2000; Li et al. 2011; MacHugh et al. 2011; Momtaz et al. 2014; Nene et al. 2012; Sinnathamby et al. 2004; Svitek et al. 2014). Two of these studies focused on predicting antigenic peptides of foot and mouth disease virus (FMDV) (Guzman et al. 2010; Momtaz et al. 2014), while additional studies examined the induction of CTL response following FMDV infection or vaccination in cattle (Childerstone et al. 1999; Guzman et al. 2008) and swine (Patch et al. 2013; Patch et al. 2011). The potential role of

CD8+ T-cell responses in FMDV infections or following vaccination remains controversial, as most FMDV vaccination research has focused on the role of virus neutralizing antibodies (Grubman et al. 2010). Evidence of CD8+ T-cell responses from the few prior studies suggests additional research is justified, as understanding MHC class I FMDV epitopes and bovine CTL responses following infection or vaccination may identify opportunities to advance FMDV vaccine development.

FMDV is a picornavirus of the *Aphthovirus* genus with a linear (non-segmented) genome of single-stranded positive-sense RNA approximately 8.2 kilobases in length (Grubman et al. 2010). The genome is translated as a single polyprotein, which is cleaved by the activity of viral proteases. The P1-2A precursor portion of full-length polyprotein includes four structural capsid proteins: VP4, VP2, VP3 and VP1. FMDV causes an acute highly contagious febrile disease with vesicular lesions in cloven-hoofed animals, including domestic cattle and swine. Infection negatively impacts milk and meat production, and outbreaks in naïve populations are characterized by rapid spread and significant morbidity. Vaccination is an important component of FMDV control in endemic countries and will be increasingly essential in response to any future outbreaks in countries or regions currently free of FMDV (Grubman et al. 2010). Adenovirus-vectored FMDV vaccine has recently received conditional approval for use in the United States. This vaccine construct contains nucleotide sequence encoding the FMDV P1-2A structural proteins and the 3C protease genes inserted into the E1 region of a replication incompetent adenovirus 5 (Ad5) vector (Brake et al. 2012; Grubman et al. 2010). Multiple species have benefited from the use of adenovirus as a vaccine vector, including

humans (human immunodeficiency virus), non-human primates (simian immunodeficiency virus), dogs (rabies), swine (porcine respiratory and reproductive syndrome virus) and cattle (bovine parainfluenza virus type 3, bovine herpes virus 1, and bovine viral diarrhea virus) (Tatsis and Ertl 2004). Human Ad5 vectors have been shown to specifically induce CD8+ T-cell responses in rodents, dogs, and nonhuman primates (Tatsis and Ertl 2004).

The bioinformatics tools, such as *NetMHCpan* and *NetMHC*, can be used to predict peptides that are likely bound by the BoLA molecules, and to inform subsequent *in vitro* and *ex vivo* studies (Hansen et al. 2014; Nene et al. 2012; Svitek et al. 2014). These tools have been used to refine the peptide predictions obtained from previous studies (Nene et al. 2012), and have been applied to successfully identify immunogenic peptides of *Theileria parva* antigens driving CD8+ T-cell responses in cattle (Svitek et al. 2014). The reverse immunology approach has been used in bacterial and viral pathogens, such as meningococcus B, human immunodeficiency virus, yellow fever virus, West Nile virus, dengue virus, herpes virus, and hepatitis C virus (Bruno et al. 2015; Rappuoli 2001).

In this study, we applied bioinformatic prediction tools, *NetMHCpan-2.9* and *MHCcluster*, combined with high throughput biochemical affinity and stability assays to identify potential FMDV epitopes bound by MHC molecules expressed by Holstein cattle in the research and teaching herd at the University of Vermont (UVM). We were able to identify several *in vitro* binding peptides and characterized the peptide motifs for five out of six BoLA molecules. This research provides insight into specific viral derived peptides

that could induce bovine CD8⁺ T-cells after infection or vaccination. This approach is a rapid and accurate method to identify T-cell epitopes and sets the stage to examine T-cell specific immune responses in Holstein cattle, other domestic cattle breeds and perhaps other bovids.

3.3. Materials and Methods

3.3.1 Animals

Whole blood samples (8-10 ml in K3-EDTA) were taken by jugular or caudal tail venipuncture from 81 purebred Holstein cattle from the UVM Dairy Center of Excellence (DCE) research herd. Blood samples were held on ice and within two hours of sampling peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque 1083 (Sigma-Aldrich, St. Louis, MO). All animal use procedures were conducted in accordance with protocol #13-059 approved by the UVM Institutional Animal Care and Use Committee.

3.3.2 RNA extraction and cDNA synthesis

RNA was purified from PBMCs using manufacturer's instructions for TRIzol Reagent in combination with RNeasy mini kit (Qiagen, Valencia, CA). Following RNA extraction, complementary DNA was generated using the ImProm-II Reverse Transcription System (Promega, Madison, WI) following manufacturer's recommendations.

3.3.3. *BoLA typing by polymerase chain reaction with sequence-specific primers (PCR-SSP)*

PCR-SSP for a panel of 15 BoLA alleles was performed according to Ellis et al., (1998) with slight modifications. Primers to amplify a 16th allele (BoLA-2*01201) were obtained from Codner (2010). PCR reaction mixtures consisted of 3.5 mM MgCl₂, 0.2 mM dNTPs, 1 μM of each primer, and 1.25 units of Taq polymerase in 1X buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂ (New England Biolabs, Ipswich, MA)) in a final reaction volume of 50 μL. Previously published annealing temperatures were adjusted for primer pairs 1, 5, 6, 7, 8, 10, 11, 13, 15, and 16, using the formula $T_m(^{\circ}\text{C}) = [67.5 + 0.34(\%GC \times 100) - 395/\text{length of the primer}]$. The cycling conditions were 95°C for 60 s followed by 30 PCR cycles of 95°C for 30 s, 55-61°C for 30 s, and 72°C for 30 s using a programmable C1000 Thermal Cycler (Bio-Rad, Hercules, California). Positive control cDNA for BoLA-3*00201, -1*02301, and -6*01301 was kindly provided by Dr. Ivan Morrison (Roslin Institute, University of Edinburgh, Edinburgh, Scotland).

3.3.4 *Positional Scanning Combinatorial Peptide Libraries and FMDV peptides*

Positional scanning combinatorial peptide library (PSCPL) peptides were synthesized using solid-phase Fmoc chemistry on 2-chlorotriyl chloride resins and purchased from Schafer-N Copenhagen, Denmark (www.schafer-n.com). Briefly, an equimolar mixture of 19 of the common Fmoc amino acids (excluding cysteine) was prepared for each synthesis and used for coupling in eight positions, whereas a single Fmoc amino acid (including cysteine) was used in one position. This position was changed in each synthesis starting with the N-terminus and ending with the C-terminus.

In one synthesis, the amino acid (AA) pool was used in all nine positions. Therefore, the library consisted of $20 \times 9 + 1 = 181$ individual peptide libraries, and includes:

- 20 PSCPL sublibraries describing position 1: AX₈, CX₈, DX₈, ... YX₈
- 20 PSCPL sublibraries describing position 2: XA X₇, XC X₇, XD X₇, ... XY X₇
- Etc.
- 20 PSCPL sublibraries describing position 9: X₈A, X₈C, X₈D, ... X₈Y
- A completely random nonamer peptide library pool: X₉,

where, X denotes the random incorporation of AA from the mixture, and a single-letter AA abbreviation is used to denote the identity of a fixed AA (e.g., A, alanine). Following synthesis, the peptides were cleaved from the resin in trifluoroacetic acid/1,2-ethanedithiol/triisopropylsilane/water 95:2:1:3 v/v/v/v, precipitated in cold diethyl ether and extracted with water before desalting on C18-columns, freeze drying, and weighing.

Peptides for the predicted FMDV CD8⁺ T-cell epitopes were synthesized by 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry, purified by reverse-phase high-performance liquid chromatography (to at least >90% purity), validated by mass spectrometry, and quantified by weight (GenScript, Piscataway, NJ).

3.3.5 Synthesis of recombinant MHC-I heavy chain proteins

Recombinant molecules BoLA-1*01901, -2*00801, -2*01601, -3*01701, -4*02401, and -6*01302 were produced as previously described (Hansen et al. 2014). In brief, each BoLA molecule was generated in *Escherichia coli* including a biotinylation substrate peptide (BSP) at the N-terminal end, which was biotinylated *in vivo* using a co-

induced BirA enzyme and the addition of biotin during the expression. *E. coli* were harvested as inclusion bodies, extracted into Tris buffered 8M urea and purified using ion exchange, hydrophobic, and gel filtration chromatography. MHC-I heavy chain proteins were never exposed to reducing conditions, which allows for purification of highly active pre-oxidized BoLA molecules when diluted into an appropriate reaction buffer. The pre-oxidized, denatured proteins were stored at -20°C in Tris buffered 8M urea.

3.3.6 Production of MHC-I light chain (beta-2-microglobulin, $\beta_2\text{m}$) protein

Native, recombinant human and bovine $\beta_2\text{m}$ were expressed and purified as previously described (Hansen et al. 2014). Briefly, a histidine affinity tag (HAT) followed by an FXa restriction enzyme site was inserted N-terminally of a synthetic gene encoding the native, mature human or bovine $\beta_2\text{m}$. The recombinant gene was expressed in the *E. coli* expression host, BL21 (DE3), harvested as inclusion bodies, extracted into a urea buffer, and purified. The tagged $\beta_2\text{m}$ protein was digested for 48 h at room temperature with the FXa protease releasing intact natively folded $\beta_2\text{m}$. The folded $\beta_2\text{m}$ was purified as previously described, and fractions containing $\beta_2\text{m}$ were identified by A_{280} UV absorbance and SDS-PAGE, and then pooled. Protein concentrations were determined by BCA assay. The native $\beta_2\text{m}$ proteins were stored at -20°C .

3.3.7 Determining peptide-MHC-I binding motifs by a scintillation proximity assay (SPA)

Nonameric peptide binding motifs were determined for BoLA-1*01901, -2*00801, -3*01701, -4*02401, and -6*01302 using the PSCPL as previously described

(Hansen et al. 2014; Harndahl et al. 2011; Rasmussen et al. 2014). Recombinant, biotinylated MHC-I heavy chain molecules in 8M urea were diluted at least 100-fold into PBS buffer containing bovine β_2m and peptide to initiate pMHC-I complex formation. The final concentration of MHC-I heavy chain was between 10-100 nM, depending on the specific activity of the MHC-I heavy chain. The reactions were carried out in the wells of streptavidin-coated scintillation 384-well Flashplate® PLUS microplates (Perkin Elmer, Waltham, MA). Recombinant bovine β_2m was expressed, purified and radiolabeled with iodine (^{125}I) as previously described (Harndahl et al. 2011). ^{125}I -labeled β_2m (approximately 1 nM, corresponding to approximately 25,000 cpm/well) and saturating concentrations (10 μ M) of peptide were allowed to reach steady state by overnight incubation at 18°C. After overnight incubation excess unlabeled bovine β_2m was added to a final concentration of 1 μ M and the temperature was raised to 37°C to initiate dissociation. pMHC-I dissociation was monitored for 24 hours by consecutive measurement of the scintillation microplate on a scintillation TopCount NTX multiplate counter (Perkin Elmer, Waltham, MA). PSCPL dissociation data were analyzed as described (Rasmussen et al. 2014). Briefly, following background correction, the area under the dissociation curve (AUC) was calculated for each sub-library by summing the counts from 0-24 hours. The relative contribution of each residue in each position (i.e., the Relative Binding, RB) was calculated as $RB = (AUC_{sublibrary} / AUC_{X9})$. The RB values were normalized for each peptide position to give a sum of 20 for each residue. The anchor position (AP) value for each peptide position was calculated as:

$$AP = \sum_{i=1}^{20} (1 - RB_{sublibrary})^2$$

Data generated by the PSCPL assay for the five BoLA molecules were used to select a library of peptides to evaluate *in vitro* binding affinity of these molecules.

3.3.8 Measuring peptide-MHC-I affinity interactions by luminescent oxygen channeling immunoassay (LOCI) AlphaScreenTM assay

A set of up to 192 peptides was selected from our peptide repository, which contains over 9,000 nonamer peptides. Based on availability in the library, up to 96 high scoring peptides (rank score ≤ 0.5) according to the PSCPL matrices were selected, and as many as possible up to 96 additional peptides were selected based on *NetMHCpan-2.8* predictions, but with low affinity according to PSCPL. This combined method has been demonstrated to successfully identify high affinity MHC binding peptides, and inclusion of such data has been shown to improve the predictive performance of the *NetMHCpan* algorithm (Hansen et al. 2014; Hoof et al. 2009; Nielsen et al. 2008; Pedersen et al. 2012; Rasmussen et al. 2014).

Affinity measurements of peptide-MHC-I interactions with MHC-I molecules were conducted using the AlphaScreenTM technology, as previously described (Harndahl et al. 2009). The donor and acceptor beads of the LOCI were purchased from Perkin Elmer (Waltham, MA). Donor beads were obtained pre-conjugated to streptavidin; acceptor beads were in-house conjugated to the monoclonal antibody W6/32 (gift from Dr. Jonathan Boyson), to a final concentration of 1 mg/ml using standard procedures as described by the manufacturer. Recombinant, biotinylated MHC-I heavy chains, human

β 2m and peptide were titrated in phosphate-buffered saline (PBS) with 0.1% Lutrol F-68 as surfactant. They were allowed to fold for 48 hours at 18°C. The folding mixture was transferred to a 384-well Optiplate (Perkin Elmer, Waltham, MA). The donor and acceptor beads were each added to a final concentration of 5 μ g/mL and incubated overnight at 18°C. To ensure temperature equilibrium during the reading time, plates were placed at room temperature one hour prior to reading on BioTek Synergy H4 (BioTek, Winooski, VT) or on an EnVision® multilabel reader (PerkinElmer, Waltham, MA). All handling of LOCI reagents was done in the dark or in green light.

LOCI signals were converted to peptide-MHC-I complex concentration via a pre-folded peptide-MHC-I complex standard. The peptide-MHC-I binding affinity was calculated by fitting peptide dose-response data to a one-site binding model ($Y=B_{\max} * X / (K_d + X)$) by non-linear regression where Y is the concentration of pMHC-I complexes formed and X is the concentration of ligand (i.e., peptide) (Harndahl et al. 2009). The EC₅₀ approximates the equilibrium dissociation constant (K_d) as long as the receptor concentration used is less than the K_d thus avoiding ligand depletion. Under conditions of limited receptor concentration ($[MHC-I HC] \leq K_d$), the EC₅₀ is a reasonable approximation of the K_d. K_d calculations were done in GraphPad Prism® 6 Software (GraphPad Software, Inc., San Diego, CA).

3.3.9 Bioinformatics analysis of the FMDV peptide binding specificity of BoLA molecules

The up-dated pan-specific algorithm *NetMHCpan-2.9* (Hoof et al. 2009) was used to predict peptide-MHC-I interactions of BoLA-1*01901, -2*00801, -2*01201, and -4*02401 with nonameric and decameric peptides of the P1 region of FMDV serotype

A24 Cruzeiro. The FMDV P1 sequence used in this analysis was obtained from Dr. Marvin Grubman (Personal Communication, Supplemental Fig. 1) and is the sequence used in construction of the new Adenoviral vectored FMDV vaccine (Brake et al. 2012).

3.3.10 Measuring specific FMDV-peptide-MHC-I interactions by LOCI and SPA

The top strong binding FMDV peptides, i.e., a *NetMHCpan* rank score of $\leq 0.5\%$ were synthesized as described above for use in subsequent binding affinity (LOCI) and stability (SPA) assays with synthetic BoLA-1*01901, -2*00801, -2*01201, and -4*02401. We also tested two weak binding peptides, i.e., those with a *NetMHCpan* rank score of 0.51-2%, thus, a total of 30 synthetic peptides were selected. Peptide-MHC stability in the dissociation SPA was expressed as a half-life in hours and, based on a previous study, we used a threshold of one hour to identify potential immunogenic epitopes (Harndahl et al., 2012).

3.3.11 Functional predictions of BoLA molecules using MHCcluster, visualized with sequence logos

MHCcluster server 2.1 was used to generate an unrooted tree and heat-map of the functional relationship between the BoLA molecules using full-length BoLA class I protein sequences, as previously described in Thomsen et al., (2013). Ovar-1-N*00201 MHC-I sequence (GI:108792434) was also included. As part of the *MHCcluster* output, a sequence logo for each allele was generated using the *Seq2Logo* server (Thomsen and Nielsen 2012). The logos are visual representations of predicted nonamer amino acid binding motifs of individual BoLA molecules. Briefly, logos were created from the

strongest binders (predicted top 1%) from a set of 100,000 random 9-mer peptides and were clustered using the Hobohm 1 algorithm with the similarity threshold of 63% to remove redundancy, and pseudo counts were applied with a weight on prior of 200 to account for a low number of observations. The accuracy of the predicted sequence motif is estimated from the distance to the “nearest neighbor” MHC molecule included in the training of the peptide binding prediction method; a value ≥ 0.70 is considered an accurate prediction and ≥ 0.90 is considered highly accurate (Hoof et al. 2009; Thomsen et al. 2013).

3.4. Results

3.4.1 BoLA class I typing of individual animals

Frequency of known BoLA gene expression was determined by PCR-SSP (Ellis et al. 1998) for 81 Holstein cattle from the UVM teaching and research herd (Figure 3-1). We used the observed frequencies in our cattle population and availability of other recombinant BoLA molecules (e.g., BoLA-2*01201 (Hansen et al. 2014)) to inform synthesis of recombinant BoLA molecules for this study. We chose to synthesize BoLA-1*01901, -2*00801, -2*01601, -3*01701, -4*02401, and -6*01302.

*3.4.2 Determining the peptide binding specificity of BoLA-1*01901, -2*00801, -3*01701, -4*02401, and -6*01302 by dissociation driven PSCPL analysis*

We applied a positional scanning combinatorial peptide libraries (PSCPLs) approach combining SPA based peptide-MHC-I dissociation assay with nonameric PSCPLs (Rasmussen et al. 2014) to analyze the peptide binding specificity of six BoLA-I

molecules: BoLA-1*01901, -2*00801, -2*01601, -3*01701, -4*02401, and -6*01302 (Table 3-1). Peptide binding motifs (Figure 3-2) and PSCPL matrices (Figure 3-5) were derived for five BoLA MHC class I heavy chains, BoLA-1*01901, -2*00801, -3*01701, -4*02401, and -6*01302. Recombinant BoLA-2*01601 was also synthesized, but we were unable to derive a peptide binding motif for this molecule due to low signal-to-background ratio in the SPA-based pMHC-I dissociation assay (data not shown). Overall, the shape of the motifs of these BoLA molecules appeared to be very similar to previously reported BoLA motifs with anchor positions at the N- and C-terminal parts of the peptide (Hansen et al. 2014). All molecules had prominent anchors in C terminal position 9 (P9) and a few motifs had a less defined primary anchor position at position 2 (P2) or position 3 (P3). The BoLA-1*01901 molecule had a pronounced anchor position in P2, P3, and P9 with strong preference for glutamic acid in P2, preference for arginine or lysine in P3, and alanine, valine or serine in P9. BoLA-2*00801 and -4*02401 have very similar peptide binding motifs; both contain less pronounced anchors in P2, but had strongly pronounced preference for aromatic amino acids in the C-terminal (P9) peptide position (Figure 3-2). The motif for BoLA-3*01701 was rather unique with potential anchor residues in P2, P3, P5, P8 and P9. BoLA-3*01701 had a strong pronounced preference for glycine in P2, and preferences for amino acids with more neutral side chains in P3. BoLA-3*01701 also appeared to favor asparagine or methionine in P5, and asparagine, proline or valine in P8. Similar to BoLA-1*01901, BoLA-3*01701 strongly favored alanine and valine in P9. The motif for BoLA-6*01302 had a distinct preference

for hydrophobic amino acids, such as leucine, phenylalanine, isoleucine, and methionine in P9 and a less pronounced P2 anchor with preference for glutamic acid and glutamine.

3.4.3 BoLA binding affinity for predicted nonameric peptides and retraining NetMHCpan for specific BoLA molecules

Peptide-MHC-I affinity interactions were measured by LOCI and these data were used to update the existing bioinformatics predictor *NetMHCpan v2.8*. In this approach, the PSCPL matrices are used to select predicted BoLA-I binding peptides from an in-house repository of >9,000 nonameric peptides. To complement this selection method, *NetMHCpan v2.8* was used to select additional strong binding peptides from the repository that were not predicted to bind according to the PSCPL derived binding matrix. We have previously demonstrated that using either the PSCPL or the *NetMHCpan* method alone is suboptimal to improve the predictive algorithm (Hansen et al. 2014; Rasmussen et al. 2014). These previous studies concluded that PSCPL matrix guided selection will be able to identify a large proportion of binders, although in many cases this will not cover the complete binding space of a particular MHC molecule. The number of predicted binders available from the nonamer peptide repository included 170, 170, 145, 170, and 157 peptides for BoLA-1*01901, -2*00801, -3*01701, -4*02401, and -6*01302 molecules, respectively (Table 3-2). For each BoLA molecule-peptide combination, the binding affinity was subsequently determined using the LOCI assay (Hansen et al. 2014; Harndahl et al. 2009). Binding affinity data was already available for BoLA-2*01201 (Hansen et al. 2014), the molecule found at the highest frequency in our herd. For BoLA-2*00801, and 6*01302 >90% of the peptides from the pool of PSCPL

matrix predicted peptides were found to bind with an affinity <500nM. Greater than 60% of the predicted peptides identified by the complementary *NetMHCpan-2.8* prediction bound at the same affinity (Table 3-2). Similarly, the PSCPL matrix based prediction for BoLA-1*01901 and -4*02401, found 31% and 40% of the predicted peptides for each molecule to bind with an affinity <500nM. The *NetMHCpan v2.8* prediction identified additional strong binding peptides (4 and 10% of peptides predicted by *NetMHCpan v2.8* were found to bind to BoLA-1*01901 and -4*02401, respectively, Table 3-2). The complementary method of PSCPL matrix plus *NetMHCpan* predictions identified a large number of MHC-I binding peptides that can in turn be used to update the prediction algorithm (Hansen et al. 2014; Rasmussen et al. 2014). One exception was BoLA-3*01701 where only 4% of the peptides predicted to bind according to the PSCPL matrix-based-prediction bound with an affinity <500nM, and the *NetMHCpan v2.8* prediction identified no additional binders (Table 3-2). The small number of binders found for BoLA-3*01701 is potentially due to the bias in the peptide repository. The prevalence of peptides matching BoLA-3*01701 motif is much lower compared to the other molecules, and in particular there are no peptides in our repository that match both preferred anchor amino acids, glycine in P2, and alanine or valine in P9. Similar results have been seen for HLA-C*0401(Rasmussen et al. 2014).

The data generated from the LOCI binding assay were used to retrain and update the *NetMHCpan* algorithm for the BoLA-1*01901, -2*00801, -2*01201, -4*02401 and -6*01302. The estimated prediction accuracies in *NetMHCpan-2.8* for these 5 molecules were 0.489, 0.437, 0.853, 0.398, and 0.809 respectively, and following retraining the

accuracies improved to 0.853 for each molecule in *NetMHCpan v2.9*. The accuracy predictions were extracted from *MHCcluster* output and were calculated from the distance to the nearest neighbor in the training data of *NetMHCpan v2.9*.

3.4.4 Identification of peptides derived from FMDV and bound by BoLA-1*01901, -2*00801, -2*01201, and -4*02401.

The updated version of *NetMHCpan v2.9* was used to make FMDV peptide predictions and is available at (<http://www.cbs.dtu.dk/services/NetMHCpan/>). The 736 amino acid fragment of FMDV P1 capsid proteins (Figure 3-4) used in this study generates a total of 1455 peptides (728 nonameric and 727 decameric peptides). Screening of these potential epitopes using a *NetMHCpan v2.9* rank score threshold of $\leq 0.5\%$ identified 28 FMDV candidate peptides binding to 4 BoLA molecules found frequently in our herd: 1*01901, 2*00801, 2*01201, and 4*02401 (Table 3-1 and Figure 3-2). We also included 2 peptides with rank score of 0.51-2%. Although functional synthetic molecules are available for BoLA-6*01302, our goal was to focus on molecules most frequently observed in our herd and FMDV peptide binding assays was not performed for -6*01302 at this time.

Among the 30 peptides, 25 (13 nonamers, and 12 decamers), were predicted to bind with strong or weak affinity to a single BoLA molecule, while five peptides ²⁴⁰MTAHITVPY₂₄₈, ⁴⁴⁴AAHCIHAEW₄₅₂, ²⁰⁷LLVAMVPEW₂₁₅, ⁴⁴³RAHCIHAEW₄₅₂, ⁶⁰⁴VVRHEGNLTW₆₁₃ were common to more than one BoLA molecule. 5 nonamer peptides were nested within decamer peptides that were predicted to bind the same molecule. Only two 10-mer peptides were predicted to be strong binders (rank score \leq

0.5) to BoLA-1*01901 allele, while thirteen peptides were predicted to bind to BoLA-2*00801 (six 9-mers, and seven 10-mers) and nine peptides were predicted as strong binders to BoLA-2*01201 (six 9-mers, and three 10-mers). Two peptides, ³⁸⁶AAKHMSNTY₃₉₄ and ²⁴⁰MTAHITVPY₂₄₈, were predicted to be weak binders (rank score > 0.5) to BoLA-2*00801 and BoLA-2*01201, respectively.

The 30 peptides were tested for *in vitro* binding affinity and stability using the LOCI and SPA assays (Table 3-1). In this study, we included 2 peptides (³⁸⁶AAKHMSNTY₃₉₄ and ²⁴⁰MTAHITVPY₂₄₈), which were predicted by NetMHCpan to be weak binders; these peptides were found to be either weak- or non-binding in the LOCI assay and had short binding half-lives (≤ 0.3 hrs.) in the SPA. In comparison, among the 28 peptides predicted to be strong binders by *netMHCpan v2.9*, the LOCI assay identified 9 strong ($K_d < 50$ nM), 5 intermediate ($K_d = 50 - 499$ nM) and 10 weak ($K_d = 500-5000$ nM) binders (Table 3-1), and 13 molecules had binding stabilities > 1 hour in the SPA. LOCI binding affinity thresholds suggest at least one weak binder ($K_d \leq 5,000$ nM) was identified for each BoLA molecule. Only two molecules, BoLA-2*00801 and -2*01201, had peptides that were predicted strong or intermediate binders, whereas only weak binding or non-binding peptides were found for BoLA-1*01901 and 4*02401. We identified 9 strong binders ($K_d < 50$ nM) for BoLA-2*00801. A SPA binding stability half-life greater than 1 hour was identified for 7, 4, and 2 peptides for BoLA-2*00801, -2*01201, and -4*02401 molecules, respectively, suggesting there may be immunogenic peptides among the predictions for these 3 molecules.

3.4.5 Functional predictions of BoLA class I allele groups

The *MHCcluster* tree was generated to predict the functional relationship of the BoLA molecules. Three large clusters were found (Figure 3-3). Structural motifs and binding specificities were predicted for all BoLA molecules. Logos highlighting the binding specificities are shown for a subset of 15 BoLA molecules (highlighted in red in Figure 3-3). Molecules predicted to have similar binding specificity clustered closely together. For example, BoLA-2*00801 and -4*02401 shared a favored binding specificity for aromatic amino acids, tryptophan, phenylalanine and tyrosine in the C-terminal peptide position. A static heat-map describing the functional relationship of the BoLA molecules is shown in figure 3-6.

3.5. Discussion

We have extended our approach for epitope discovery, including immunoinformatics and biochemical peptide binding affinity and stability assays, to identify candidate FMDV class I MHC restricted epitopes in Holstein cattle. Previous work using this approach has focused on epitope prediction for *T. parva* antigens (Hansen et al. 2014; Nene et al. 2012; Svitek et al. 2014). Three observations have emerged from these prior studies. First, peptides that have been previously identified as epitopes from experimental studies are frequently identified as potential epitopes by the *NetMHCpan* predictor. Second, the predictor will often identify alternative peptides that are predicted to have higher binding affinity compared to CD8+ T-cell epitopes previously identified in experimental studies, and these alternatives are frequently shorter peptides nested within the previously identified CD8+ T-cell epitope (Nene et al. 2012). Third, the process of

using epitope predictions to guide *in vitro* and *ex vivo* experiments generates new and sometimes unanticipated data that can be used to iteratively inform improvements in the *NetMHCpan* and subsequent live animal immune response studies.

Only a fraction of peptides that bind to MHC molecules are CD8+ T-cell epitopes, with potential epitopes first narrowed by MHC class I determinants and further by TCR repertoire restriction and antigen processing (Yewdell and Bennink 1999). In order to be considered a CD8+ T-cell epitope, the pMHC complex must trigger a specific T-cell immune response. Peptide affinity and stability are two factors that play an important role in determining the immunogenicity of a peptide (Busch and Pamer 1998; Harndahl et al. 2012). Positional scanning combinatorial peptide libraries (PSCPLs) have successfully been applied to address peptide-MHC-I specificity of humans (Rasmussen et al. 2014; Stryhn et al. 1996), swine (Pedersen et al. 2012), and cattle (Hansen et al. 2014). In cattle epitopes or peptide binding motifs have been characterized for at least 13 BoLA molecules (Gaddum et al. 1996a; Gaddum et al. 1996b; Graham et al. 2007; Graham et al. 2008; Hansen et al. 2014; Hegde et al. 1999; Hegde et al. 1995; Li Pira et al. 2010; Macdonald et al. 2010; MacHugh et al. 2011; Momtaz et al. 2014; Nene et al. 2012; Svitek et al. 2014), and we have expanded characterization of peptide binding motifs for five additional molecules. We also produced recombinant BoLA-2*01601, but this molecule had a poor β_2m complex-specific signal in the SPA using the PSCPL and predicted FMDV specific peptides were not tested in the LOCI or SPA assays. Possible reasons for this non-functional molecule could be poor quality of the protein preparation or intrinsic biochemical properties of the molecule that result in incompatibility with the

assay. For example, it is possible this particular molecule has a preference for 8-mers, 10-mers or 11-mers instead of 9-mers used in the PSCPL, which would explain why a motif could not be derived (Stevens et al. 1998). Alternative approaches, such as peptide elution experiments, might be considered for molecules that appear to be incompatible with *in vitro* binding assays. While we were able to generate data for BoLA-3*01701 using the PSCPL, subsequent testing in the SPA was unsuccessful, possibly due to loss of protein function, and specific FMDV peptide binding data was not generated in the LOCI or SPA. Another potential reason for the difficulty in characterizing the peptide binding affinity of BoLA-3*01701 could be due to a relatively limited peptide binding repertoire outside of the PSCPL. Further work to identify peptide motifs and epitopes for these two molecules is warranted as both appear to occupy regions of the *MHCcluster* diagram that lack *in vivo* or *in vitro* data supporting motif predictions (Figure 3-3). For example, BoLA-3*01701 estimated prediction accuracy is 0.47 using nearest neighbor Rhesus macaque Mamu-B3901 and this molecule has been previously described as potentially not conforming to more commonly found peptide motifs (De Groot et al. 2003). Again, peptide elution experiments might be the most effective method to characterize the peptide motif for BoLA-3*01701. In comparison, for BoLA-6*01301 where *in vitro* and *in vivo* data are available from eight different studies (Gaddum et al. 1996b; Graham et al. 2006; Graham et al. 2008; Guzman et al. 2008; Hansen et al. 2014; Macdonald et al. 2010; Nene et al. 2012; Svitek et al. 2014), the estimated prediction accuracy is 0.853. While we did not identify BoLA-6*01301 in our herd, we did identify BoLA-6*01302 in 25% of UVM-DCE Holstein cattle (Figure 3-1). A higher frequency of 6*01302 relative

to 6*01301 has also recently been reported in other cattle populations (Svitek et al., 2015). A PSCPL matrix was derived for 6*01302; however, at this time we elected to complete *in vitro* binding assays for the four other molecules more frequently identified in our herd. BoLA-2*01201 was previously synthesized and a motif was generated (Hansen et al. 2014), and we decided to test FMDV predicted peptides for this molecule because it was found in relatively high frequency in our herd.

NetMHCpan-2.9 predicted 28 peptides derived from the FMDV structural proteins that would most likely bind four common BoLA molecules (BoLA-1*01901, -2*00801, -2*01201, and -4*02401) identified among animals from our research herd. *NetMHCpan* is trained on human, mice, swine, and a handful of BoLA peptide-MHC binding data. It is capable of making accurate predictions where no binding data are available (Nielsen et al. 2008). In our current study, 50% (14/28) of the peptides predicted by the *NetMHCpan-2.9* algorithm with a rank score $\leq 0.5\%$ were demonstrated to have high or intermediate binding affinity in the LOCI assay. The number of predicted peptide binders for a particular molecule is affected by the rank cut-off value selected. For example, the algorithm predicted only two strong binding peptides for BoLA-1*01901, and by increasing the rank threshold cutoff to greater than 2%, we could potentially identify more binders. However, previous benchmark studies determined the vast majority of epitopes have binding with a rank score of 2% or less (Erup Larsen et al. 2011). Therefore, applying a *NetMHCpan* threshold of 2% allowed us to reduce the number of FMDV capsid protein 9- and 10-mer peptides from over 700 to 10 and 14 for alleles BoLA-2*01201 and -2*00801, respectively. Results of the binding assays allow us

to further narrow the pool of peptides that might be evaluated as potential epitopes in T cell response assays for vaccinated or virus challenged cattle, especially cattle expressing BoLA-2*00801 where we observed 7 peptides with binding stability half-lives > 1 hour. The limited number of potential epitopes identified for the remaining molecules could either be a function of a limited number of epitopes in FMDV structural proteins or current limitations in the predictive models.

BoLA-2*00801 and -4*02401 share 92% sequence identity in the peptide binding groove, and were predicted to have similar peptide binding specificities. The algorithm predicted four FMDV peptides (⁴⁴⁴AAHCIHAEW₄₅₂, ²⁰⁷LLVAMVPEW₂₁₅, ⁴⁴³RAAHCIIHAEW₄₅₂, ⁶⁰⁴VVRHEGNLTW₆₁₃) as potential binders to both BoLA-2*00801 and -4*02401. However, our *in vitro* results indicate that these peptides had strong affinity for BoLA-2*00801 and weak affinity for BoLA-4*02401. A potential reason for the differences in binding could be due to the subtle differences in the key amino acids in the anchor positions. Both molecules have common amino acids, tryptophan and phenylalanine in P9, however, the P2 anchor appears less important for BoLA-4*02401. The amino acids in P2 for BoLA-4*02401 have aromatic hydrophobic side chains, as opposed to BoLA-2*00801, which has amino acids with polar, neutral side chains in this position (Figure 3-2). This subtle difference could be the reason for weak binding by BoLA-4*02401. As more BoLA binding data are generated, the algorithm can be iteratively retrained to capture these differences.

Perhaps more importantly, these data can be used to inform *ex vivo* CTL response or CD8+ tetramer assays using cells from cattle expressing these BoLA molecules. For

example, Svitek et al., (2014) recently used tetramer assays to identified a *T. parva* epitope (Tp5₈₇₋₉₅) that binds to two BoLA molecules (-1*02301 and -T5). The role of cattle CD8⁺ T-cell responses to FMDV infection or vaccination remains controversial and a limited number of studies have evaluated class I restricted CD8⁺ T-cell responses following FMDV infection or vaccination (Childerstone et al. 1999; Guzman et al. 2010; Guzman et al. 2008; Patch et al. 2013; Patch et al. 2011). An important next step is to test whether the peptides identified in this study are MHC-I epitopes in cattle expressing the BoLA molecules evaluated in this study. For example, the CD8⁺ response to peptides identified in this study could be evaluated among cattle vaccinated with the AD5-FMDV vaccine expressing A24 P1 capsid proteins.

Thus far, it appears that leucine, phenylalanine, isoleucine, and methionine are important anchor residues in P9 of the BoLA class I binding motif. Position two is more variable, with glutamic acid, glutamine, leucine, arginine, threonine, and serine being the most commonly favored amino acids in this anchor position. In one specific cluster, tryptophan was identified as a strong anchor residue in P9. In another cluster, lysine and arginine were identified as favored anchor residues in P9. Some motif predictions have been validated in animals. For example, the CD8⁺ T-cell antigen Tp2 and epitope ⁴⁹KSSHGMGKVGK₅₉ from the Muguga strain of *T. parva*, matched the binding specificity for BoLA-2*01201 predicted by the algorithms (Nene et al. 2012). In contrast, MHC cluster did not capture the P5 anchor of the BoLA-6*01301 molecule previously identified to bind an immunogenic peptide (Macdonald et al. 2010). This is not surprising as the P5 anchor appears to be relatively rare and these rare events are less well

recognized by the predictor tool. We also find it worth noting that peptides without the P5 anchor appear to have strong binding affinities for BoLA-6*01301 and -6*01302 and these peptides are immunogenic (Hansen et al. 2014; Svitek et al. 2015).

The functional relationship between all known BoLA molecules was predicted using the *MHCcluster* algorithm. The characterization of the BoLA binding specificities may also make it possible to cluster molecules that share largely overlapping peptide binding specificities (i.e., BoLA supertypes) (Lund et al. 2004; Sidney et al. 2008). A MHC cluster tree generated following inclusion of our data shows there are currently two clusters lacking binding data; representative molecules from each of these clusters are highlighted in blue (Figure 3-3). The predictions of the *NetMHCpan v2.9* and *MHCcluster* algorithms can be improved by characterizing the binding specificity of additional BoLA molecules, perhaps by first addressing regions of the tree currently lacking coverage.

In conclusion, we have translated technologies that have been applied to the study of human T-cell responses to the study of cattle immunity. These technologies include bioinformatics, peptide synthesis, and proteomics. Using FMDV or other important pathogens, extending this research should advance our understanding of how viral antigens are recognized by the bovine immune system, and contribute to improved vaccine development against bovine pathogens.

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3.7. Conflict of Interest

The authors declare that they have no conflict of interests in the publication of this work.

3.8. References

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3.9. Figures and Tables

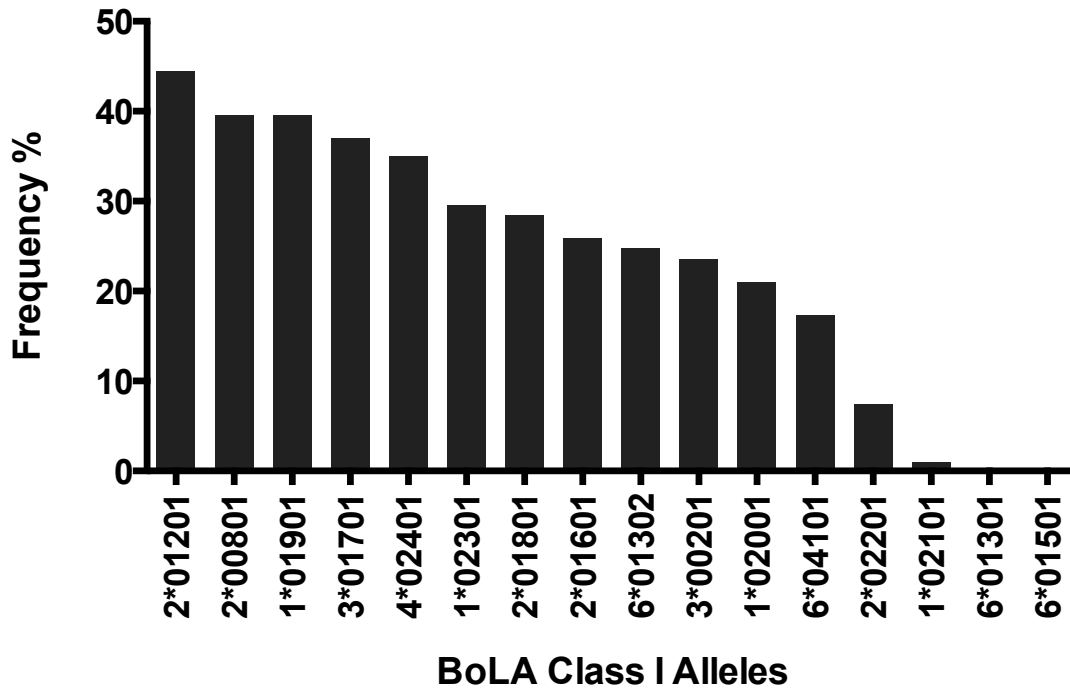


Figure 3-1: Frequency of BoLA class I alleles in 81 Holstein cattle from the UVM Dairy Center of Excellence research herd assayed by PCR-SSP.

Table 3-1: FMDV (strain A24) P1 region peptides predicted as strong (n=28) or weak (n=2) binders using NetMHCpan v2.9.

Table 1. 30 peptides predicted as strong or weak binders using NetMHCpan v2.9 from the P1 region of FMDV strain A24						
BoLA molecule	Peptide Sequence ^a	NetMHCpan Predicted % rank score ^b	kD (nM)	Actual binding status ^c	pMHC-I complex stability ^d	
1*01901	²⁰⁰ NQFNGGCLLV ₂₀₉	0.5	1086	Weak	0.3	
	⁴⁹⁹ AENDTLVVSV ₅₀₈	0.12	7817	Non-binder	0.5	
2*00801	⁶⁰ TTNTQNDW ₆₈	0.4	9	Strong	2.3	
	⁶⁵³ TVYNGTSKY ₆₆₁	0.4	5	Strong	1.5	
	¹¹⁴ SSVGVTHGY ₁₂₂	0.15	6	Strong	0.8	
	⁴⁴⁴ AAHCIHAEW ₄₅₂	0.25	21	Strong	17.8	
	²⁰⁷ LLVAMVPEW ₂₁₅	0.3	2	Strong	6.1	
	⁴⁴³ RAAHCIIAEW ₄₅₂	0.08	1.7	Strong	5.4	
	⁶⁰⁴ VVRHEGNLTW ₆₁₃	0.15	11	Strong	1.1	
	⁵⁹ HTTNTQNDW ₆₈	0.3	40	Strong	0.2	
	¹⁸² DSYAYMRNGW ₁₉₁	0.25	2	Strong	2.7	
	²⁴⁰ MTAHITVPY ₂₄₈	0.25	51	Intermediate	0.8	
	³³² KVYNPPRTNY ₃₄₁	0.5	393	Intermediate	0.6	
	³⁸⁶ AAKHMSNTY ₃₉₄	1.5	1299	Weak	0.2	
	³⁸⁵ LAAKHMSNTY ₃₉₄	0.5	1380	Weak	0.2	
	²⁸⁴ KVYANIAPTY ₂₉₃	0.4	>10,000	Non-binder	0.2	
	2*01201	⁶⁸⁵ ASFNYGAIK ₆₉₃	0.01	67	Intermediate	4.6
⁶²⁶ TSNPTAYNK ₆₃₄		0.25	226	Intermediate	2.2	
³²⁴ KTADPAYGK ₃₃₂		0.25	443	Intermediate	6.0	
⁴⁹⁰ CIYQITHGK ₄₉₈		0.5	1035	Weak	6.8	
³⁷¹ RTDDTRLAK ₃₈₀		0.4	4176	Weak	0.3	
¹⁴⁰ RVVQAERFYK ₁₄₉		0.4	1353	Weak	0.6	
¹⁴¹ VVQAERFYK ₁₄₉		0.25	>20,000	Non-binder	0.2	
²⁵⁰ GVNRYDQYK ₂₅₈		0.5	8693	Non-binder	0.5	
²⁴⁰ MTAHITVPY ₂₄₈		1.5	5547	Non-binder	0.3	
⁶³⁰ TAYNKAPFTR ₆₃₉		0.4	>20,000	Non-binder	0.3	
4*02401	⁴⁴⁴ AAHCIHAEW ₄₅₂	0.2	572	Weak	2.7	
	²⁰⁷ LLVAMVPEW ₂₁₅	0.15	588	Weak	0.5	
	⁴⁴³ RAAHCIIAEW ₄₅₂	0.08	1792	Weak	1.7	
	⁶⁰⁴ VVRHEGNLTW ₆₁₃	0.4	1001	Weak	0.4	

^aPeptide positions are relative to the peptide sequence shown in supplemental figure 2

^b9mer or 10mer peptides predicted as strong binders (rank score ≤ 0.5) or weak binders (rank score > 0.5) using NetMHCpan v2.9

^cActual binding status according to the LOCI assay. Strong binder (Kd < 50 nM), intermediate binder (Kd 50-500nM), weak binder (Kd 501-5000nM), non-binder (Kd > 5000 nM)

^dPeptide binding stability half-life in hours

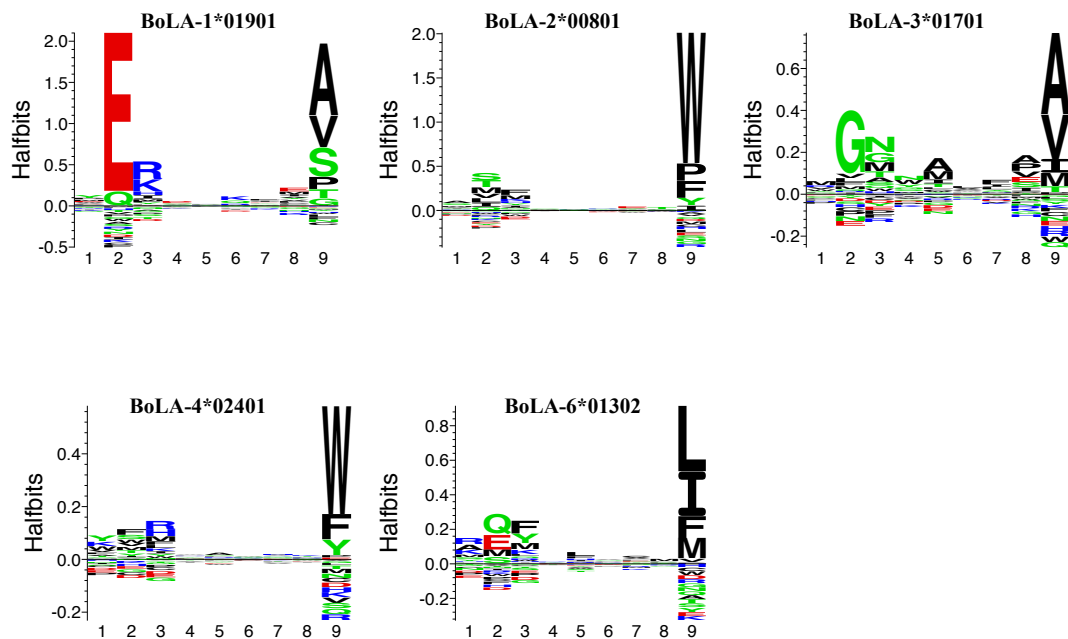


Figure 3-2: Sequence logo representations of BoLA-1*01901, 2*00801, 4*02401, 6*01302 binding motifs. The logo for BoLA-2*01201 has been previously described (Hansen et al. 2014). The logos were calculated from the top 1% highest scoring peptides selected from a pool of 9-mer peptides using the positional scanning combinatorial peptide library matrix. In the sequence logo, each peptide position is represented by a stack of letters indicating its significance for binding and the height of each amino acid is proportional to its relative frequency (Thomsen and Nielsen 2012). Acidic residues are displayed in *red*, basic in *blue*, neutral in *green*, and hydrophobic in *black*.

>FMDV-P1-A24

MGAGQSSPATGSONQSGNTGSIINNYMQQYQNSMDTQLGDNAISGGSNEGST
DTTSTHTTNTQNNDWFSKLASSAFTGLFGALIADKKTEETTLEDRLTTRNGHT
TSTTQSSVGVTHGYSTEEDHVAGPNTSGLETRVVQAERFYKKYLFDWTTDKAFG
HLEKLELPSDHHGVFGHLVDSYAYMRNGWDVEVSAVGNQFNGGCLLVAMVPE
WKEFDTREKYQLTLFPHQFISPRTNMTAHITVPYLGVNRYDQYKKHKPWTLVV
MVVSPLTVNNTSAAQIKVYANIPTYVHVAGELPSKEGIFPVACADGYGGLVTT
DPKTADPAYGKVYNPPRTNYPGRFTNLLDVAEACPTFLCFDDGKPYVTTRTDDT
RLLAKFDLSLAAKHMSNTYLSGIAQYYTQYSGTINLHFMFTGSTDSKARYMVAY
IPPGVETPPDTPERAACHIAEWDTGLNSKFTFSIPYVSAADYAYTASDTAETINV
QGWVCIYQITHGKAENDTLVVSVSAGKDFELRLPIDPRQQTATGESADPVTTTV
ENYGGETQIQRRHHTDIGFIMDRFVKIQLSPTHVIDLMQTHQHGLVGALLRAAT
YYFSDLEIVVRHEGNLTWVPNGAPESALLNTSNPTAYNKAPFTRLALPYTAPHR
VLATVYNGTSKYAVGGSGRRGDMGSLAARVVKQLPASFNYGAIKADAIHELLV
RMKRAELYCPRPLLAIEVSSQDRHKQKIIAPAKQ

Figure 3-4: Amino acid sequence of FMDV-P1 region from serotype A24.

BoLA-1*01901	Amino acid position in peptide								
	1	2	3	4	5	6	7	8	9
A	1.1	0.2	0.7	1.3	1.3	1.2	1.1	1.4	4.9
C	1.0	0.3	0.0	1.0	1.1	0.8	1.2	1.2	0.2
D	1.2	0.4	0.4	1.5	1.0	0.5	0.7	0.8	0.0
E	1.4	9.6	1.0	1.5	1.0	0.6	1.2	1.8	0.0
F	1.2	0.3	1.3	1.0	1.0	0.9	1.5	1.2	0.2
G	0.6	0.2	0.2	1.3	1.1	0.9	0.5	0.2	1.6
H	0.9	0.1	1.5	1.0	1.0	0.9	1.1	0.3	0.1
I	0.6	0.3	1.5	0.7	1.0	0.6	1.4	1.4	1.1
K	0.3	0.4	2.4	0.6	1.3	2.1	0.5	0.4	0.0
L	0.8	0.8	1.1	0.8	0.7	0.8	1.2	1.3	0.6
M	1.4	0.9	1.3	1.0	1.0	1.1	1.0	1.5	0.9
N	0.9	0.3	0.7	0.9	1.0	0.9	0.6	0.4	0.6
P	1.5	0.6	0.2	0.6	1.2	0.8	1.1	1.3	2.0
Q	0.4	2.4	0.8	1.1	0.6	1.8	1.1	0.7	0.0
R	0.6	0.5	2.9	1.0	1.1	1.5	0.5	0.3	0.0
S	1.0	0.5	0.5	1.3	0.9	1.0	1.0	1.1	3.0
T	0.7	0.6	0.3	0.9	0.8	0.8	1.1	1.0	1.7
V	1.3	0.6	0.9	0.7	0.8	1.0	1.1	1.3	3.1
W	1.3	0.5	1.1	0.7	1.1	1.1	0.9	0.9	0.1
Y	1.8	0.3	1.3	1.1	1.0	0.9	1.2	1.3	0.1
Sum	20	20	20	20	20	20	20	20	20.01
AP	3	82	10	2	1	3	2	4	34

BoLA-2*00801									
Amino acid position in peptide									
	1	2	3	4	5	6	7	8	9
A	1.7	0.9	1.2	1.2	1.2	1.0	1.2	1.1	0.1
C	0.9	0.5	0.0	1.0	1.2	0.8	1.1	0.9	0.2
D	0.4	0.4	0.5	1.1	1.0	0.8	0.7	0.8	0.1
E	0.6	0.3	0.4	1.1	0.9	0.8	1.7	1.1	0.5
F	0.9	0.5	1.8	0.9	0.9	0.9	0.9	1.0	2.4
G	1.2	0.8	0.7	1.1	1.1	1.1	1.0	1.0	0.4
H	0.9	0.6	1.4	1.0	1.2	1.3	1.0	0.8	0.5
I	1.2	1.7	1.3	0.7	0.9	1.0	0.9	0.8	1.5
K	1.0	0.5	1.4	1.0	1.0	0.9	1.0	1.0	0.0
L	1.1	1.8	1.0	0.8	0.9	0.9	1.1	0.9	0.5
M	1.3	2.0	1.6	0.9	0.9	0.8	0.9	0.9	0.5
N	0.6	0.5	1.2	1.1	1.3	0.9	1.0	0.9	0.3
P	0.4	0.4	0.6	1.0	1.0	1.1	1.0	1.3	2.4
Q	0.8	1.6	1.1	1.1	0.8	1.3	1.0	0.9	0.1
R	1.2	0.5	1.0	1.1	1.1	1.4	1.0	0.9	0.4
S	1.5	2.2	0.9	1.2	1.0	1.0	1.3	1.1	0.5
T	1.0	2.1	0.9	1.1	0.8	1.0	1.1	1.9	0.0
V	1.3	1.8	1.0	0.9	1.0	1.0	0.9	0.9	0.1
W	0.8	0.6	0.5	0.7	0.9	0.8	0.5	0.8	7.7
Y	1.1	0.4	1.3	1.0	0.9	1.2	0.7	1.0	1.7
Sum	20	20	20	20	20	20	20	20	20
AP	2	9	4	0	0	1	1	1	57

BoLA-3*01701	Amino acid position in peptide								
	1	2	3	4	5	6	7	8	9
A	1.1	1.0	1.4	0.8	2.1	1.2	0.7	1.8	3.9
C	0.8	0.6	0.1	0.8	1.2	1.2	1.1	1.2	0.6
D	0.9	0.1	0.6	0.6	0.4	0.8	0.6	1.0	1.0
E	0.8	0.3	0.5	1.0	0.3	0.8	1.0	1.6	0.6
F	0.7	1.2	0.5	1.1	0.9	1.1	1.7	0.7	0.7
G	0.8	3.6	1.7	0.7	0.6	0.8	1.0	0.4	0.9
H	0.7	0.7	1.2	0.9	1.1	1.0	1.1	0.4	0.2
I	1.0	1.1	0.8	0.8	1.4	0.8	1.4	1.4	1.7
K	0.9	1.0	1.0	0.5	0.9	0.7	0.6	0.5	0.1
L	1.2	1.3	1.1	0.9	1.2	1.5	1.4	0.8	0.8
M	1.6	1.2	1.7	1.2	1.7	1.0	1.1	0.8	1.7
N	1.1	0.3	2.0	1.6	0.4	0.8	0.9	0.6	0.6
P	0.4	0.5	0.3	0.5	0.7	1.1	1.0	1.8	1.1
Q	1.1	1.1	1.3	1.3	0.8	1.1	1.2	0.7	0.4
R	1.3	1.1	0.4	1.1	0.8	0.7	0.7	0.5	0.3
S	0.8	0.8	1.3	1.3	1.0	0.7	0.9	1.6	0.8
T	1.0	0.9	1.5	1.0	1.4	1.0	0.9	1.1	1.3
V	1.3	1.3	0.9	0.9	0.8	1.3	0.6	1.6	2.8
W	1.3	1.0	1.2	1.6	1.2	1.2	0.8	0.7	0.3
Y	1.2	0.8	0.6	1.4	1.1	1.1	1.3	0.9	0.1
Sum	20	20	20	20	20	20	20	20	20
AP	2	9	5	2	4	1	2	4	17

BoLA-4*02401	Amino acid position in peptide								
	1	2	3	4	5	6	7	8	9
A	1.2	1.1	1.0	0.8	1.5	1.0	1.2	1.1	0.9
C	0.7	0.5	0.2	0.9	0.9	0.7	0.8	1.1	0.6
D	0.6	0.3	0.6	1.1	1.0	0.8	0.9	0.8	0.6
E	0.5	0.6	0.5	0.9	0.8	0.8	1.1	1.0	1.1
F	1.2	1.5	1.3	1.2	1.2	1.0	1.1	0.8	2.1
G	1.1	0.5	0.5	1.1	1.2	0.9	1.1	1.0	0.9
H	0.8	0.6	1.6	1.1	1.1	1.1	1.3	1.2	0.6
I	0.8	1.2	1.2	0.7	1.1	1.0	0.8	0.8	1.0
K	1.5	0.8	1.2	0.8	1.0	1.1	0.8	1.3	0.6
L	0.9	1.0	1.3	1.1	0.9	1.0	0.8	1.2	0.9
M	1.1	1.3	1.4	1.0	0.9	1.0	1.2	1.0	0.7
N	0.8	0.9	1.1	1.0	1.0	1.1	1.1	0.8	0.6
P	0.5	1.1	0.7	1.1	1.1	0.9	0.9	1.0	1.1
Q	0.7	1.1	0.8	1.1	0.9	1.2	1.1	1.0	0.4
R	0.9	0.8	1.6	1.1	1.2	1.1	1.1	1.1	0.4
S	1.1	1.4	0.9	0.9	1.0	1.0	1.1	0.9	0.3
T	1.2	1.3	0.8	0.8	0.9	1.1	0.8	1.2	0.7
V	1.3	1.3	0.9	1.1	0.9	1.1	0.7	0.9	0.3
W	1.4	1.4	1.2	1.2	0.7	1.2	1.0	0.9	4.4
Y	1.6	1.3	1.2	1.1	0.8	1.0	1.1	0.9	1.7
Sum	20	20	20	20	20	20	20	20	20
AP	2	2	3	0	1	0	1	0	16

BoLA-6*01302	Amino acid position in peptide								
	1	2	3	4	5	6	7	8	9
A	1.6	0.9	1.1	0.9	1.0	0.8	1.3	1.0	0.3
C	0.8	0.6	0.0	1.0	1.0	1.3	1.3	1.2	0.6
D	0.4	0.4	0.4	0.9	0.9	0.9	1.0	0.9	0.5
E	0.4	2.3	0.6	1.1	0.8	0.9	1.2	1.0	0.3
F	1.0	0.5	2.0	0.9	1.4	1.2	1.2	0.9	2.3
G	1.0	1.1	0.4	1.0	1.0	0.9	1.2	0.7	0.5
H	1.1	0.5	1.1	1.0	1.3	1.1	1.0	0.9	0.6
I	1.0	0.7	1.1	0.8	1.3	0.9	1.0	1.1	3.2
K	1.5	0.7	1.4	1.1	0.9	0.9	0.6	0.8	0.4
L	1.1	1.0	1.2	1.1	1.7	1.1	0.8	1.2	4.0
M	1.3	1.7	1.5	1.0	1.2	1.0	0.6	1.6	2.2
N	0.5	0.8	0.9	1.0	1.0	0.8	0.8	0.9	0.2
P	0.3	0.6	0.5	0.9	0.9	0.6	1.1	1.1	1.1
Q	0.8	2.7	1.2	1.2	1.0	1.3	0.9	1.1	0.3
R	1.7	0.7	1.2	1.3	1.2	1.1	0.6	0.8	0.5
S	1.3	1.7	0.6	1.0	0.6	0.8	0.9	1.1	0.5
T	0.8	0.8	0.8	0.9	0.6	0.9	1.1	0.9	0.3
V	1.1	0.8	1.0	1.0	0.7	1.3	1.3	1.1	1.3
W	1.0	0.6	1.2	0.8	0.7	1.2	1.0	0.8	0.5
Y	1.2	1.0	1.8	1.1	0.7	1.1	1.0	1.0	0.3
Sum	20	20	20	20	20	20	20	20	20
AP	3	7	4	0	2	1	1	1	22

Figure 3-5: PSCPL Matrices of BoLA-1*01901, -2*00801, -3*01701, -4*02401 and -6*01302 determined by SPA. Relative binding values >2 are considered favored amino acids, values 0.5-1.5 are considered tolerated and values below 0.5 are considered disfavored. AP values > 10 are considered anchor positions. The sequence logos were generated using the Seq2Logo server (Thomsen and Nielsen 2012).

Table 3-2: Distribution of peptide-BoLA-I binding affinity for peptides predicted to bind according to either PSCPL matrix based prediction or NetMHCpan prediction (of peptides with low-scoring PSCPL matrix values).

Binding affinity (k _d , nM)	BoLA-1*01901		BoLA-2*00801		BoLA-3*01701		BoLA-4*02401		BoLA-6*01302	
	PSCPL	NetMHCpan	PSCPL	NetMHCpan	PSCPL	NetMHCpan	PSCPL	NetMHCpan	PSCPL	NetMHCpan
<50	1 (1%)	1 (1%)	69 (86%)	34 (38%)	3 (4%)	0 (0%)	1 (1%)	1 (1%)	74 (95%)	32 (41%)
50-499	27 (30%)	3 (4%)	7 (9%)	26 (29%)	0 (0%)	0 (0%)	30 (39%)	9 (10%)	3 (4%)	24 (30%)
500-5,000	34 (37%)	14 (18%)	3 (4%)	21 (23%)	4 (5%)	0 (0%)	25 (32%)	15 (16%)	0 (0%)	18 (23%)
>5,000	29 (32%)	61 (77%)	1 (1%)	9 (10%)	67 (91%)	71 (100%)	21 (27%)	68 (73%)	1 (1%)	5 (6%)
Number of peptides	91 (100%)	79 (100%)	80 (100%)	90 (100%)	74 (100%)	71 (100%)	77 (100%)	93 (100%)	78 (100%)	79 (100%)

**4. CHAPTER 4: INDUCTION OF A CD8+ T CELL RESPONSE WITH
ADENOVIRAL VECTOR BASED FMDV VACCINE ENCODING P1
LINKED TO THE MHC CLASS II CHAPERONE PROTEIN INVARIANT
CHAIN**

This manuscript has been formatted for Veterinary Research.

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4.1. Abstract

Foot and mouth disease (FMD) is a highly infectious and economically devastating viral disease of livestock. Recent vaccination strategies are directed primarily toward the induction of neutralizing antibody responses. Current FMD virus (FMDV) vaccines have yielded partial successes, eradicating disease in some areas of the world and delaying disease in others. However, several challenges of vaccine design still remain such as short duration of immunity and serotype-specific protection. The primary goal of this study was to improve vaccine performance by targeting cellular immunity. Here, we have used a recombinant human adenovirus vector to deliver the P1 capsid of FMDV tethered to the bovine major histocompatibility complex (cattle MHC; BoLA) class II chaperone protein invariant chain (Ii) to investigate the CD8⁺ T cell responses in cattle. The vaccine construct induced a CD8⁺ T cell response that was measured by IFN- γ release. This response was directed towards two epitopes, ⁶⁵³TVYNGTSKY₂₁₅ and ²⁰⁷LLVAMVPEW₂₁₅, within the P1 capsid proteins of FMDV strain A24. In an attempt to show MHC class I restriction peptide-MHC tetramers were generated; however, no tetramer specific staining of T cells was detected. Our results show that identification of these epitopes is important in not only the analysis of the cellular immune responses, but also the potential to improve FMDV vaccine performance by targeting cellular immunity.

Keywords: IFN- γ ELIspot; Tetramers; Major Histocompatibility Complex class I; Cattle; Adenovirus

4.2. Introduction

Foot-and-mouth disease (FMD) is an economically and agriculturally important disease of cattle and other cloven-hoofed animals and is enzootic to all continents except Australia and North America [1, 2]. The highly infectious nature of the virus requires strict control measures to ensure reduced and restricted disease transmission. Current control strategies include vaccinating animals with chemically inactivated whole-virus in adjuvant vaccines, mass culling (stamping out) of susceptible animals in outbreaks and restrictions on the exportation of contaminated animals or animal products. Both stamping out and trade restrictions can have devastating economic effects, as seen in the 2001 UK outbreak which resulted in the culling of over 4 million animals and a loss of \$12-13.8 billion dollars [3, 4]. Outbreaks such as this highlight the need for better prevention, biosecurity and control measures, including improved vaccines.

FMD inactivated antigen vaccines have been in existence for more than 70 years, these vaccines, though partially effective, have several limitations. First, the current FMDV killed virus vaccines achieve serotype and subtype specific protection in 1-2 weeks, but fail to induce long-term protective immunity, thus revaccination is often required every 4 to 6 months [5, 6]. Production and manufacture of inactivated antigen vaccines requires growth of large quantities of virus in biosafety level 3 (specialized containment) facilities, virus inactivation and purification [7, 8]. Vaccines usually have a

short shelf life and cold-chain requirements [5]. Additionally, to differentiate infected from vaccinated animals (DIVA), vaccine production requires the removal of cellular contaminants as well as nonstructural viral proteins in order to support DIVA, this is essential for maintaining trade status [5].

Such vaccine challenges can be addressed by gaining a better understanding of the overall immune response to FMDV vaccination and infection. Generally, vaccination and infection induces a humoral response, generating serotype specific neutralizing antibodies which protect animals from clinical disease caused by homologous virus [1]. The virus has seven serotypes (A, O, C, Asia 1, and South African Territories 1, 2 and 3) and multiple subtypes occur within each serotype [1]. This is a problem for vaccine design, as the vaccine strain needs to match the circulating strain of the virus. FMDV infection is not always controlled solely with neutralizing antibodies, therefore other immune cells such as CD8⁺ T cells could have an important role in FMDV vaccine design and protection.

CD8⁺ T cells are involved in controlling viral infections and recognize antigens presented by the major histocompatibility complex (MHC) class I molecules (Citation). Upon antigen recognition naïve CD8⁺ T cells differentiate into effector T cells, which recognize and kill infected cells thru two pathways such as direct cytotoxicity or release of cytokines such as IFN- γ (Citation). Several studies have explored the role of T cell mediated immune responses to FMDV in cattle and swine following infection or vaccination [9-19]. A few of these studies have demonstrated CD8⁺ T cell MHC class I

restriction in cattle and swine in FMDV [12, 13, 15, 17-19]. Despite these studies, the significance of the CD8⁺ T cells in protection and vaccination remain unclear.

Over the past decade improvements in vaccine technology have helped address several FMD vaccine limitations. One such example was the development of an FMDV vaccine that is delivered by a replication defective human adenovirus serotype 5 vector (Ad5-FMD) [20]. Unlike the traditional killed virus vaccines, Ad5-FMD vaccine does not require large amounts of virus for production, is DIVA compatible and is protective in both cattle and swine [20]. However, similar to the traditional killed virus vaccines, neutralizing antibodies generated to the Ad5-FMD vaccine strain are serotype specific and do not provide protection across serotypes, or subtypes within serotypes [5].

Recently, Patch et al., developed a recombinant human Ad5-FMD vectored vaccine containing a mutation in the 3C protease ($3C^{pro}$) to illicit a T cell directed immune response in swine [15]. They hypothesized this vaccine enhanced cytolytic activity by preventing $3C^{pro}$ from cleaving the capsid proteins, thus targeting all the FMDV proteins for degradation and increasing antigen presentation on MHC class I. It was demonstrated that in the absence of significant humoral immunity, a cytotoxic T cell (CTL) response could reduce clinical disease and decrease viremia in swine challenged with live virus [14]. Therefore, the combination of humoral and cellular immunity may be able to diminish outbreaks of FMDV especially when the infecting viruses are poorly matched to the serotype-specific vaccine strains available.

Previous studies in mice with an adenoviral vaccine vector system which expressed the glycoprotein of lymphocytic choriomeningitis virus tethered to the MHC

class II invariant chain (Ii), showed an enhanced immunogenicity to CD4+ and CD8+ T cells [21-26]. In this proof of concept study, we have expanded upon these previous findings from bovine, swine and mice to study the CD8+ T cell response in cattle. We have designed a recombinant human Ad5 vector containing the P1 capsid of FMDV tethered to the bovine leukocyte antigen (Cattle MHC; BoLA) class II invariant chain lacking the 3C^{pro}, which we designate as Ad5-P1-Ii. Our hypothesis was that an intradermal vaccination with the Ad5-P1-Ii will generate a FMDV specific CD8+ T cell response by targeting proteins for degradation and presentation on MHC class I that can be measured by tetramer staining and IFN- γ release. Here we report after vaccination with Ad5-P1-Ii, two FMDV epitopes ⁶⁵³TVYNGTSKY₂₁₅ and ²⁰⁷LLVAMVPEW₂₁₅ elicited an IFN- γ response by CD8+ T cells.

4.3. Materials and Methods

4.3.1. Animals

Four Holstein steers (176, 182, 184, 185) 4-6 months old and weighing 300-500 lbs were housed at the Miller research farm at the University of Vermont Dairy Center of Excellence (UVM-DCE). Animals were maintained according to UVM-DCE established practice. All animal procedures were reviewed and approved by University of Vermont Institutional Animal Care and Use Committee (protocol #13-059).

4.3.2. Cell lines

Human 293 cells (ATCC CRL-1573) were used to generate, grow and titer all recombinant human Ad5 vectors used in this study. Cells were maintained in MEM (Life

Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 1% L-glutamine (Life Technologies, Grand Island, NY), and 1% Penicillin-Streptomycin (Life Technologies, Grand Island, NY) Peripheral blood mononuclear cells (PBMCs) and CD8 T cells used in the ELISPOT assay were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT), 1% L-glutamine (Life Technologies, Grand Island, NY), 1% Penicillin-Streptomycin (Life Technologies, Grand Island, NY) and 0.1% 2-mercaptoethanol (Life Technologies, Grand Island, NY). Swine kidney cells (IBRS2) were used in Western blot and maintained in DMEM supplemented with 10% FBS (Hyclone, Logan, UT), 1% L-glutamine, 1% nonessential amino acids (Life Technologies, Grand Island, NY) and 1% Penicillin-Streptomycin (Life Technologies, Grand Island, NY). Baby hamster kidney (BHK-21) cells (ATCC CCL-10) were used in Western blot and maintained in basal medium Eagle (BME) (Life Technologies, Grand Island, NY) supplemented with 10% FBS, 10% tryptose phosphate broth (Life Technologies, Grand Island, NY), 1% L-glutamine (Life Technologies, Grand Island, NY), and 1% Penicillin-Streptomycin (Life Technologies, Grand Island, NY).

4.3.3. Vaccination

Three Holstein steers (176, 184 and 185) were vaccinated with 3×10^9 PFU Ad5-P1-Ii in PBS. PBS was administered to one additional Holstein steer (182) as a negative control. Animals were boosted with 1.25×10^9 PFU Ad5-P1-Ii in PBS. Initial dose of virus was delivered intradermally in a total of 3 ml to three sites on the neck (1.0 ml/site) using

a pulse injection system (Pulse NeedleFree systems, Lenexa, KS). For the boost, virus was delivered intradermally in a total of 5 ml to five sites on the neck (1.0 ml/site) using the pulse injection system (Pulse NeedleFree systems, Lenexa, KS).

4.3.4. Isolation of PBMCs and CD8 T cells

Whole blood and serum were collected from all four steers by jugular or caudal tail venipuncture into sodium heparin and serum vacutainer tubes (BD Biosciences, San Jose, CA) on days 0, 7, 14, 21 and 28 days post vaccination. Eleven weeks following initial vaccination, steers were boosted and samples were collected on days 0, 4, 7, 11, 14, 21 and 28 post boost. 0.03mg/kg of xylazine (Lloyd Lab, Decatur, IL) was administered intravenously at all time points where whole blood was collected. PBMCs were isolated using Uni-Sep Maxi tubes (Novamed, Jerusalem, Israel). Briefly, 14 ml of whole blood was aliquoted into each Uni-Sep tube, and then centrifuged at 1000g for 20 min at room temperature. The band of PBMCs was harvested and washed three times with 1xPBS (Life Technologies, Grand Island, NY), followed by suspension in complete RPMI. To positively select CD8⁺ T cells, purified PBMCs were labeled with monoclonal mouse anti-bovine CD8 ILA-105 (3µg/ml) followed by secondary goat anti-mouse IgG2a-PE (at 4µg/ml) (ThermoFisher Scientific, Suwanee, GA). Followed by MACS goat anti-mouse PE microbeads and separated on LS columns using a MidiMACS magnetic separator (Miltenyi Biotec, Gladbach, Germany), according to the manufacturer's instructions.

4.3.5. Virus production

The bovine invariant chain sequence (accession: NM_001034735.1) was used to design a fusion of invariant chain with the P1 region of FMDV strain A24, with invariant chain at the N-terminus. 4 µg of DNA containing the FMDV-P1-Ii was synthesized by Bio-Basic (Bio-Basic Inc, Amherst, NY). 100 µl of ddH₂O was used to dissolve the DNA. Transformation was done using chemically competent *E. coli* cells as described previously [27]. Briefly, 2 µl of FMDV-P1-Ii DNA was added into a vial of *E. coli* cells, gently mixed, and incubated on ice for 30 minutes. Cells were then heat-shocked for 30 seconds at 42°C and immediately transferred to ice. 250 µl of room temperature SOC medium was added to the cells, after which they were shaken horizontally (200 rpm) at 37°C for 1 hour. The mixture was plated on Luria-Bertani (LB) plates (Teknova, Hollister, CA) containing 50 µg/ml of Ampicillin and incubated overnight at 37°C. Five colonies were picked and inoculated into 5 mls of LB broth containing 50 µg/ml of ampicillin and maintained overnight in a shaking incubator at 37°C.

Plasmid DNA was isolated using PureLink™ Quick Plasmid Miniprep Kit (Life Technologies, Grand Island, NY). The plasmid DNA underwent a restriction digest using *ClaI* and *XbaI*, and the product was run on 1% agarose gel to visualize the insert (2851 bp). The band of interest was excised from the gel and purified using Qiaquick® gel purification kit (Qiagen, Valencia, CA). Plasmid Ad5-Blue was obtained from Dr. Marvin Grubman (Agricultural Research Service, USDA). The purified construct was ligated into the pAd5-Blue vector that had been digested with *ClaI* and *XbaI*, as previously described [27]. The final plasmid was linearized with *PacI* and 4 µg of DNA

was transfected into Human Embryonic Kidney (HEK) 293 cells (seeded two days prior to transfection) using Lipofectamine 2000 (Life Technologies, Grand Island, NY) at an insert to vector ratio of 3:1. The recombinant virus was harvested 4 days post transfection.

Virus was extracted from the HEK293 cells by three consecutive freeze/thaw cycles and amplified by infection of a larger culture of HEK293 cells [27]. The virus was purified using Adenopure Kit (Puresyn, Malvern, PA). Virus titer was established by determining the tissue culture infectious dose 50 (TCID₅₀). Briefly, 10-fold serial dilutions of virus in MEM were added to HEK 293 cells in a 96-well flat bottom microtiter plate, ten replicates per dilution. Tissue culture plates were incubated at 37°C for 10 days, and monitored for cytopathic effect (CPE) in order to calculate the TCID₅₀.

4.3.6. Western blotting

IBRS2 cells or BHK cells were seeded in a 6-well plate at 1×10^6 cells/well in 3 ml supplemented MEM with 10% fetal bovine serum (Hyclone, Logan, UT), 1% non-essential amino acids (Life Technologies, Grand Island, NY) and 1% Penicillin-Streptomycin (Life Technologies, Grand Island, NY) incubated for 24 h at 37°C. Following attachment, IRBS2 cells were infected with adenovirus Ad5-P1-Ii or Ad5-Blue (negative control), BHK cells were infected with FMDV-A24 (positive control) at a multiplicity of infection (MOI) of 20 and then incubated for 48 h in a 37°C incubator. Whole-cell lysates were harvested and lysed with cell lysis buffer (150mM NaCl, 10mM Tris pH 7.6, 1% NP40, 1% DOC) and stored at -80°C until further use. 18 µl of cell lysates were mixed with 9 µl of loading dye and were run under denaturing conditions on

12% Bis-Tris SDS-PAGE gels (Life Technologies, Grand Island, NY) and transferred onto nitrocellulose membranes using Novex mini-cell XCell SureLock electrophoresis system (Life Technologies, Grand Island, NY). The blots were probed with FMDV-specific polyclonal antibodies (mAbs) VP0, 1 and 3 cocktail (1:2000) to detect P1. Secondary antibodies were goat α -rabbit IgG (1:2000) (KPL, Gaithersburg, Maryland) for the VP cocktail. Pre-stained protein ladder was used as the molecular weight marker (ThermoFisher Scientific, Suwanee, GA).

4.3.7. Tetramer construction

Peptides and recombinant BoLA-2*00801 and bovine β 2-microglobulin were produced and complexed into tetramers as described previously [28]. Briefly, 0.25 μ M of purified BoLA heavy chain (HC) was incubated with the following: β 2m [1.5 μ M], peptide [4 μ M], EDTA (1 mM), pepstatin A (1 μ M), 1.10 phenantroline (100 μ M), TLCK (50 μ g/mL), TPCK (100 μ M), PMSF (100 μ M), Lutrol (0.03%), 50 mM of Tris-maleate (pH6.6), in a final volume of 500 μ L at 18 °C for 48 h. High molecular weight complexes were removed by centrifugation with an Amicon® Ultra Centrifugal Filter Units (Life Technologies, Grand Island, NY) at 14,000 g for 10 min. The flow-through was recovered and the p-MHC class I complexes were tetramerized at room temperature by incubating them with a total of 1.5 μ g of phycoerythrin (PE)- streptavidin (BD Biosciences, San Jose, CA) starting with 0.45 μ g and then adding 0.21 μ g every ten min over a 50 min period at room temperature and then stored at 4°C.

4.3.8. Flow cytometry

1x10⁶ bovine PBMCs were pelleted and resuspended in a blocking solution containing mixture of heat inactivated 5% rat serum (Sigma-Aldrich, St. Louis, MO) and 5% mouse serum (Sigma-Aldrich, St. Louis, MO) in PBS for 20 min at room temperature. Cells were then washed in twice in PBS, and resuspended in 10 µl of LIVE/DEAD® fixable yellow cell stain (Life Technologies, Grand Island, NY). Cells were incubated for 30 min at 4°C, washed twice in cold PBS, resuspended in 10 µl of tetramer and incubated at room temperature for 20 minutes. After incubation cells were washed twice in cold FACS buffer (PBS + 0.5% BSA), and resuspended in FACS buffer containing CD8 monoclonal antibody ILA51 (IgG₁) and CD4 antibody mouse α-bovine CD4-FITC (AbD Serotec, Raleigh, NC) and then incubated at room temperature for 30 min. Cells were washed twice in cold FACS buffer and resuspended in secondary antibody rat α-mouse IgG₁ BV421 (BD Biosciences, San Jose, CA) and incubated at room temperature for 30 min. Cells were washed twice as above, and fixed using Cytotfix/Cytoperm kit (BD Biosciences, San Jose, CA). Cells were read on the Attune® NxT Acoustic Focusing Cytometer (Life Technologies, Grand Island, NY). All cells were gated on Live cells/CD8+/Tetramer +, 100,000 events were recorded.

4.3.9. Serum neutralization assay

The measurement of endpoint serum neutralization titers of FMDV A24 were done as recently described [15, 29]. Briefly, 4-fold heat inactivated serum dilutions were incubated at 37°C with either FMDV serotype A24 or Ad5-P1-Ii suspension in cell growth medium (50 µl) was added to each well at 100 TCID₅₀ of FMDV serotype A24

for 1 hour in a microtiter plate. Cells (BHK for FMDV or HEK 293 for Ad5) from near confluent culture were enumerated and diluted in cell culture medium to 1×10^5 cells/ml, then 100 μ l of cell suspension was added to all wells. The plates were incubated at 37°C for 3 days (FMDV) or 10 days (Ad5-P1-Ii), wells were scored for cytopathic effects and endpoint titers were calculated using the Spearman-Kärber method, expressed as \log_{10} of the reciprocal of the dilution of serum that protects 50% of the wells. Titers above 1.2 \log_{10} were considered positive for anti-adenovirus antibodies while titers above 0.9 \log_{10} were considered positive for anti-FMDV antibodies [20].

4.3.10. ELISpot

MultiScreen-IP filter plates MAIPS4510 (EMD Millipore, Billerica, MA) were pre-wetted with 150 μ l 70% ethanol. The plates were washed five times with sterile water, and then coated overnight at 4°C with 5 μ g/ml monoclonal α -bovine IFN- γ antibody (MT17.1) (Mabtech, Cincinnati, OH). The bovine IFN- γ ELISpot kit was used according to manufacturer's instructions (Mabtech, Cincinnati, OH). Two fold dilutions of either 2×10^5 T cells/well or 1×10^6 PBMCs/well were plated in duplicate and 1 μ M of peptide was added. The following FMDV peptides were used: 60 TTNTQNNDW $_{68}$, 653 TVYNGTSKY $_{661}$, 444 AAHCIHAEW $_{452}$, 207 LLVAMVPEW $_{215}$, 443 RAAHCIIHAEW $_{452}$, 604 VVRHEGNLW $_{613}$, 182 DSYAYMRNGW $_{191}$, and 284 KVYANIPTY $_{293}$. Cells plated with media alone and irrelevant peptide 84 KVYANIPTY $_{293}$ was used as negative controls, as it was a non-binder in the biochemical affinity and stability assays [30]. Spots were counted and analyzed with a Zeiss KSL ELISpot automatic reader using the KSL ELISpot v2.5 software.

4.3.11. Statistical analyses

Spots from media only wells were subtracted from all experimental wells. IFN- γ ELIspot results are shown as the mean \pm SD of normalized spots per 2×10^5 CD8+ T cells. Mean and standard deviation were calculated using Microsoft Excel (Microsoft). ANOVA followed by one-way Dunnett's test was conducted in SPSS (IBM) to compare the mean IFN- γ spots generated by CD8+ T cells in response to cells pulsed with peptide (relevant versus irrelevant). IFN- γ responses needed to exceed the threshold set as the mean responses of irrelevant peptide plus twice the SD.

4.4. Results

4.4.1. Ad5-P1-Ii construct results in reduced capsid processing and production of anti-FMDV antibodies

Previous work by Patch et al., demonstrated that vaccination of swine with Ad5A24 containing a mutation (C163S) of the 3C^{pro} had reduced processing of P1 into mature capsid proteins [15]. We therefore created a second adenovirus construct with the same P1 polyprotein derived from FMDV strain A24, but removed the 3C^{pro}, which is involved in processing the viral protein. This construct was then tethered to the BoLA class II invariant chain, which we designated as Ad5-P1-Ii. In order to demonstrate the impaired activity of 3C^{pro} in the new construct, IBRS2 cells and BHK cells were infected with Ad5-P1-Ii, Ad5-Blue (empty vector) or FMDV strain A24. Cell lysates were analyzed by SDS-PAGE followed by Western blotting (Figure 4-1). We detected the

presence of P1 cleavage products, VP0 or VP1/3 in the lysates of cells infected with FMDV. Cells infected with Ad5-P1-Ii showed more unprocessed P1 (Figure 4-1). The empty vector (Ad5-Blue) was negative for an anti-FMDV antibody response.

Sera from steers vaccinated and boosted with Ad5-P1-Ii were tested for adenovirus or FMDV neutralizing antibodies as described in materials and methods. All three steers (176, 184, 185) vaccinated with Ad5-P1-Ii generated adenovirus neutralizing antibodies following initial vaccination, with elevated titers following the boost (Figure 4-2). In contrast, FMDV neutralizing titers in these three animals could only be detected following the boost (Figure 4-3). As expected, the steer (182) vaccinated with PBS did not have any detectable adenovirus or FMDV neutralizing antibodies (Figure 4-2 and 4-3).

4.4.2. Induction of a virus specific CD8⁺ T cell response in FMDV vaccinated steers

The animals in this study were previously MHC class I typed by PCR using sequence specific primers (PCR-SSP), cloning, Sanger sequencing and Illumina MiSeq v2.0 (Table 4-1) [30]. We selected these four steers based on the presence of BoLA-2*00801. This allele was found in relatively high frequency (40%) of Holstein cattle in our herd [30]. In addition we previously performed FMDV peptide-MHC affinity and stability assays with this target molecule to identify potential FMDV CD8⁺ T cell epitopes [30].

At each time point following the initial vaccination or boost, CD8⁺ T cells or PBMCs were freshly isolated and pulsed with individual FMDV peptides identified from the previous affinity and stability assays for between 18-20 hours at 37°C. The following

day activation of these cells was measured by IFN- γ ELISpot assay (Figure 4-4 and Figure 4-6). Peptide ($^{284}\text{KVYANIPTY}_{293}$) previously identified as a non-binder in the affinity and stability assays was used as a negative control in the ELISpot assay [30]. Very few PBMCs produced IFN- γ in response these peptides (data not shown). However, two peptides, $^{653}\text{TVYNGTSKY}_{215}$ and $^{207}\text{LLVAMVPEW}_{215}$, induced a statistically significant ($p = 0.001$ and $p = 0.007$, respectively) number of IFN- γ producing CD8+ T cells in one vaccinated animal (184) (Figure 4-4 and 4-5). These peptides also stimulated a slight IFN- γ response in steer 185, but these results were not statistically significant. No statistically significant response was detected in the third vaccinated steer (176) or naïve steer (182).

4.4.3. FMDV peptide-BoLA class I tetramer staining was not detected

In an attempt to show MHC class I restriction, we developed peptide-BoLA tetramers for the BoLA molecule expressed by these animals, BoLA-2*00801. Based on previous biochemical affinity and stability assays, we identified 7 potential FMDV epitopes from P1 that were used to create peptide-BoLA tetramers [30]. As a negative control, an eighth BoLA tetramer containing a non-binding peptide ($^{284}\text{KVYANIPTY}_{293}$) identified previously was used as a negative control. PBMCs were stained with a live/dead marker, antibodies specific for CD4 and CD8 T cells and with tetramer (data not shown). Cells were gated on live/CD4-/CD8+/tetramer+ cells. For all steers vaccinated with Ad5-P1-Ii no tetramer staining was detected from all samples taken after the initial vaccination or following boost.

4.5. Discussion

In the present study, we hypothesized vaccination of cattle with a T cell directed adenovirus vaccine for FMDV (Ad5-P1-Ii) would elicit a CD8⁺ T cell response that could be measured by tetramer staining and IFN- γ release. This vaccine lacked the 3C^{pro}, which prevents P1 capsid protein from being processed. Similar to a study conducted in swine, we hoped this would allow for an increase in presentation of peptides on MHC class I molecules to CD8⁺ T cells [15]. We previously identified 7 FMDV peptides that bound BoLA-2*00801 with strong affinity and stability as determined by biochemical affinity and stability assays [30]. We aimed to verify these 7 peptides as potential CD8⁺ T cell epitopes induced by vaccination with Ad5-P1-Ii. CD8⁺ T cells are attractive targets for enhancing FMDV immunity because they are stimulated by epitopes that are linear, extremely small and have the potential to be highly conserved across the various serotypes of FMDV [15]. The presence of the serum-neutralizing titers to the adenovirus vector were seen 7-21 days after the initial vaccination and again following boost, which indicates the animals successfully responded to the vaccination. In contrast, FMD virus-neutralizing antibodies in the serum were only detected following the boost.

CD8⁺ T cells combat viral infection primarily through two mechanisms: direct killing of virus infected cells or release of cytokines such as IFN- γ [31]. One experimental assay often used to detect (*in vitro*) direct killing of virus-infected cells is the cytotoxic T cell (CTL) killing assay. However, due to two main constraints, the need for genetically matched MHC class I alleles of both effector and target cells, as well as the cytopathic nature of FMDV, we employed an ELISpot assay to measure IFN- γ release

by CD8⁺ T cells from vaccinated animals in response to stimulation with FMDV specific peptides. We showed that a CD8⁺ T cell response could be detected 4 days post boost (Figure 3a). Prior to the boost, CD8⁺ T cell responses were not detected in any of the animals. (Supplemental Figure 1). The level of response varied between the three vaccinated steers, one non-responder (176), one mid-responder (185) and one high-responder (184). Two of the animals (184 and 185) had a biologically relevant response on day 4 post boost. This provides evidence to validate our proof of concept study, since these animals had a detectable response to the T cell directed Ad5-P1-Ii vaccine.

Although the response was not as expected, the findings are similar to previous studies, the frequency of CD8⁺ T cells responding to FMDV post vaccination was very low [12, 13, 15]. Our findings are also very similar to the study conducted in swine, where the CD8⁺ T cell response was only detected on day 4 post boost [15]. Similar to other picornaviruses the main focus of the immune response has been on the development of neutralizing antibodies; however, T cell responses are induced and can confer some degree of protection against virus challenge. One example is shown with type B coxsackieviruses (CVB) infection. Depletion of CD8⁺ T cells during CVB infection leads to increase in viral titers, suggesting CD8⁺ T cells may have a role in controlling the virus [32]. Another example is that infection with Theiler's murine encephalomyocarditis virus (TMEV) results in strong CD8⁺ T cell responses for which several epitopes have been identified [32]. These studies suggest that although CD8⁺ T cells are not commonly found to be major players in combating picornaviral infection, they do play an important role.

The vaccine in this proof of concept study did not contain an adjuvant; the addition of one could have induced a more pronounced CD8+ T cell response. Two examples of adjuvants that can induce a strong cytotoxic CD8+ T cell response and increase the primary immune response by activating and recruiting antigen presenting cells include saponins such as Quil A and cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) [33].

The animals in this study were not challenged with FMDV; therefore the immunogenicity of these peptides in terms of clinical protection from disease is yet to be determined. Without challenge, the induction of a sufficient CD8+ T cell response is difficult to quantify. We do not know if the FMDV peptide specific responses detected would be translated into protection or reduced viral load in the cattle, or the sufficient amount of memory needed to create an appropriate systemic immune response. Future studies should include a FMDV challenge of the vaccinated animals as well as addition of an appropriate adjuvant in order to measure the expansion of CD8+ T cells post challenge. In addition staining cells with a cytotoxicity marker such as CD107a or measuring proliferation with Ki-67 or CFSE dyes are other viable options to determine if the CD8+ T cells were proliferating and differentiating in response to vaccination or infection.

A reverse immunology approach (analyzing the key antigenic peptides presented by BoLA 2*00801 of interest) to determine FMDV peptides used in this study limited the peptide pool. Therefore, we propose future studies should synthesize the complete overlapping peptide library (15 amino acids in length and overlapping by 10 amino acids)

spanning the FMDV serotype A24 structural polyprotein P1 to capture any potential epitopes that may have been missed.

Although the response to the epitopes identified was varied, we explored whether they were conserved across the other serotypes of FMDV (Supplemental Table 1).

²⁰⁷LLVAMVPEW₂₁₅ was conserved across serotypes A22/IRAQ, Asia-1, Type C, Type O1/BFS and Type O1/UK. It was not conserved among the SAT 1, 2 and 3 serotypes.

⁶⁵³TVYNGTSKY₂₁₅ was not conserved among the other serotypes, except A22/IRAQ; however, it was previously described as having strong affinity to swine leukocyte antigen (SLA)-I*0401[34]. It is important to identify identical or similar epitopes across the different serotypes of FMDV in terms of vaccine design, as a vaccine containing these epitopes has the potential to protect against multiple FMDV serotypes.

MHC class I restriction of the virus specific CD8+ T cells responding in the IFN- γ ELISpot assay was not supported by BoLA class I tetramer staining of PBMCs derived from these animals, as no tetramer specific staining was observed. Two explanations for the lack of tetramer positive cells are discussed here. First, the sensitivity of the tetramer assay was too low to detect the number of positive T cells. ELISpot assays are more sensitive, as they have a lower detection limit of 0.0001%, which exceeds the detection limit of intracellular cytokine staining and tetramers [35]. Second, there was no true tetramer positive control (i.e., cells expressing the specific T cell receptor of interest). One method to generate a tetramer positive control is the mixed lymphocyte peptide culture method (MLPC), which uses peptide stimulation and cytokine culture to induce a specific T cell response [36]. Another method is development of a transgenic mouse

expressing BoLA genes and T cell receptor of interest [37]. These BoLA mice can be immunized with FMDV antigens to generate an epitope specific T cell response. Regarding the current study, logistical restrictions and the complexity of the BoLA limited the feasibility of this option. Finally, a third method is to use a tetramer of a different specificity that is positive in cattle, however, this is an assay control and not a tetramer positive control. For example, BoLA class I tetramers to measure *Theileria parva* specific T cell responses were recently described and could be used for this purpose [28]. An alternative method to tetramer staining to demonstrate MHC restriction of IFN- γ producing cells is to use MHC matched target cells. For example, Guzman et al., transfected P815 cells with single MHC class I alleles and then used these cells as target cells in an ex-vivo IFN- γ ELISpot assay [12, 13]. We decided not to pursue this option and focused our efforts on tetramer staining, as this was a more novel approach.

Our results demonstrated a CD8⁺ T cell response specific for FMDV epitopes measured by IFN- γ release after vaccination with T cell directed adenovirus vaccine (Ad5-P1-Ii). Previous studies have correlated an increase in IFN- γ production with reduced pharyngeal FMDV replication [38]. However, the link between the FMDV CD8⁺ T cell response and protection against disease still remains to be determined. Our future studies include addition of an adjuvant in the T cell directed adenovirus vaccine and further characterization of CD8⁺ T cells by proliferation and measurement of cytotoxicity after FMDV vaccination and infection. Ultimately, the ideal FMDV vaccine will induce both a rapid humoral and cellular immune response, achieve cross protection

against many serotypes and generate long-lasting protective immunity, thus providing insight into ways to ultimately improve vaccine performance.

4.6. List of abbreviations

Foot and Mouth Disease (FMD)

FMD virus (FMDV)

Bovine major histocompatibility complex (BoLA)

Major histocompatibility complex (MHC)

Differentiate infected from vaccinated animals (DIVA)

Human adenovirus serotype 5 vector (Ad5-FMD)

3Cprotease (3C^{pro})

Cytotoxic T cell (CTL)

Invariant chain (Ii)

Bovine leukocyte antigen (BoLA; cattle MHC)

University of Vermont Dairy Center of Excellence (UVM-DCE)

Peripheral blood mononuclear cells (PBMCs)

Fetal bovine serum (FBS)

Roswell Park Memorial Institute (RPMI)

Swine kidney cells (IBRS2)

Baby hamster kidney (BHK-21) cells

Basal medium Eagle (BME)

Cytopathic effect (CPE)

Human Embryonic Kidney (HEK)

Polyclonal antibodies (mAbs)

Phycoerythrin (PE)

Polymerase chain reaction-sequence specific primers (PCR-SSP)

Granulocyte macrophage colony-stimulating factor (GM-CSF)

Type B coxsackieviruses (CVB)

Theiler's murine encephalomyocarditis virus (TMEV)

4.7. Competing interests

The authors declare they have no competing interests.

4.8. Authors' contributions

MP, WG and JB designed the study. MP generated the vaccine construct, isolated PBMCs, isolated T cells, performed the ELISpot and tetramer experiments, analyzed the data, and drafted the manuscript. TS and KE isolated PBMCs and performed the ELISpot assay. TS performed the SVN assay. MP, MK and TdLS performed the western blot. JB and MP vaccinated the cattle. WG and JB participated in coordination and design of the study, contributed to final modifications of the manuscript. All authors read and approved the final manuscript.

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4.11. Figures and Tables

Table 4-1: Allelic profile of steers used in this study

Table 1 Allelic profile of steers used in this study

BoLA Allele	Steer 176	Steer 182	Steer 184	Steer 185
1*00901	I	I		I
1*01901^a	P/C	A	P/C	P/C
1*02001^a	C	P	P/C	P/C
1*02301^a	A			
1*03101	I	I	I	I
2*00801^a	P/I	P/I	P/I	P/I
2*01201^a			P	
2*01601^a	I	I		I
2*02601			I	C/I
2*03201N			I	I
3*00201^a	I	I		
3*01701^a		I		I
3*02702	I	I	C/I	C/I
4*02401^a	A	A	I	I
6*01302^a		P		
6*01402		I		
6*01501^a			P	P
NC1*00201	I	I		
NC1*00301	C	C	C	
NC1*00501	I			

Typing methods are abbreviated: PCR-SSP (P), Cloning & Sanger sequencing (C), Illumina MiSeq v 2.0 (I), all three methods (A)

^aBoLA alleles with PCR-SSP primers available

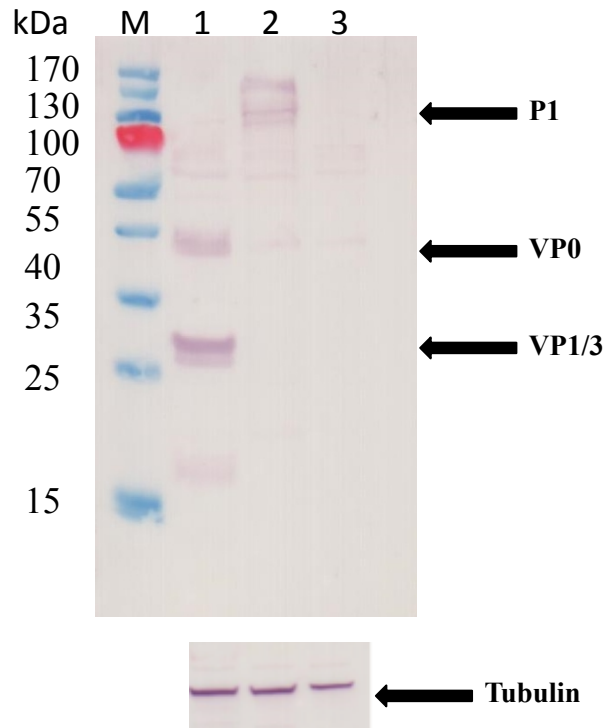


Figure 4-1: Impaired capsid processing of Ad5-FMDV-BIC. IBRS2 cells were infected with the indicated virus as described in materials and methods. Following infection, cell lysates were made and proteins were separated by SDS-PAGE and visualized by Western blotting using a mix of rabbit polyclonal antibodies against FMDV capsid proteins. M: Marker, Lane 1: Ad5-A24, Lane 2: Ad5-FMDV-BIC, Lane 3: Ad5-Blue

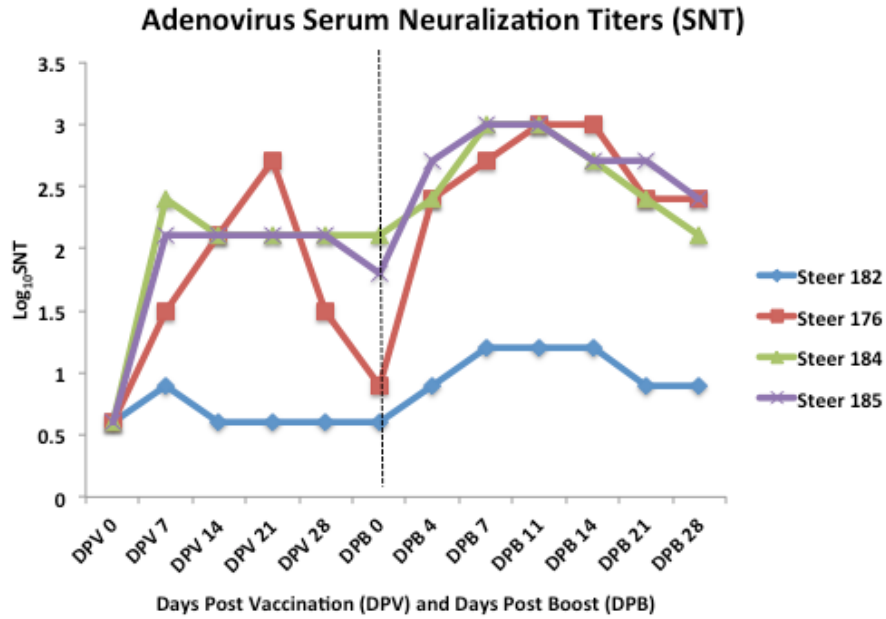


Figure 4-2: Adenovirus Serum Neutralizing Titers (SNT) from steers vaccinated with PBS or Ad5-P1-Ii. Serum samples were collected following initial vaccination (DPV=days post vaccination) and following boost (DPB=days post boost). Blue line is naïve steer (182). Red, green and purple lines represent SNT titers for 176, 184, and 185 respectively. SNT were determined as described in materials and methods. Dotted line indicates time of boost.

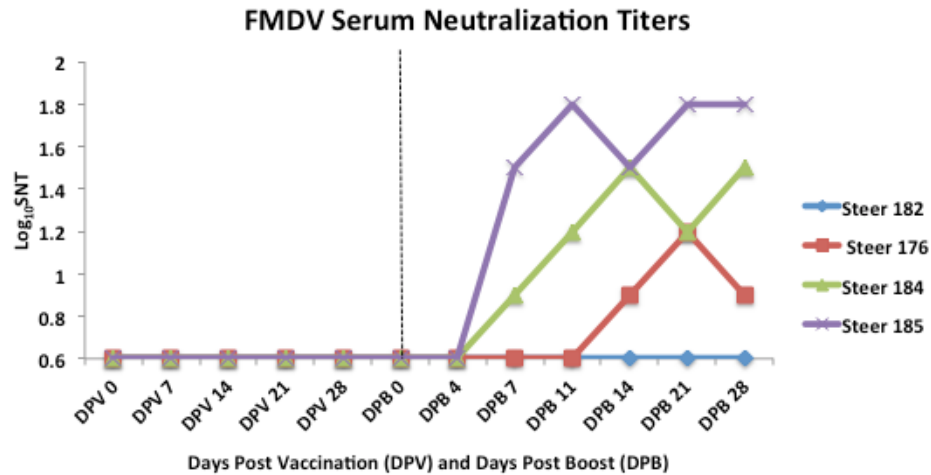


Figure 4-3: FMDV Serum Neutralizing Titers (SNT) from steers vaccinated with PBS or Ad5-P1-Ii. Serum samples were collected following initial vaccination (DPV=days post vaccination) and following boost (DPB=days post boost). Blue line is naïve steer (182). Red, green and purple lines represent SNT titers for 176, 184, and 185 respectively. SNT were determined as described in materials and methods. Dotted line indicates time of boost.

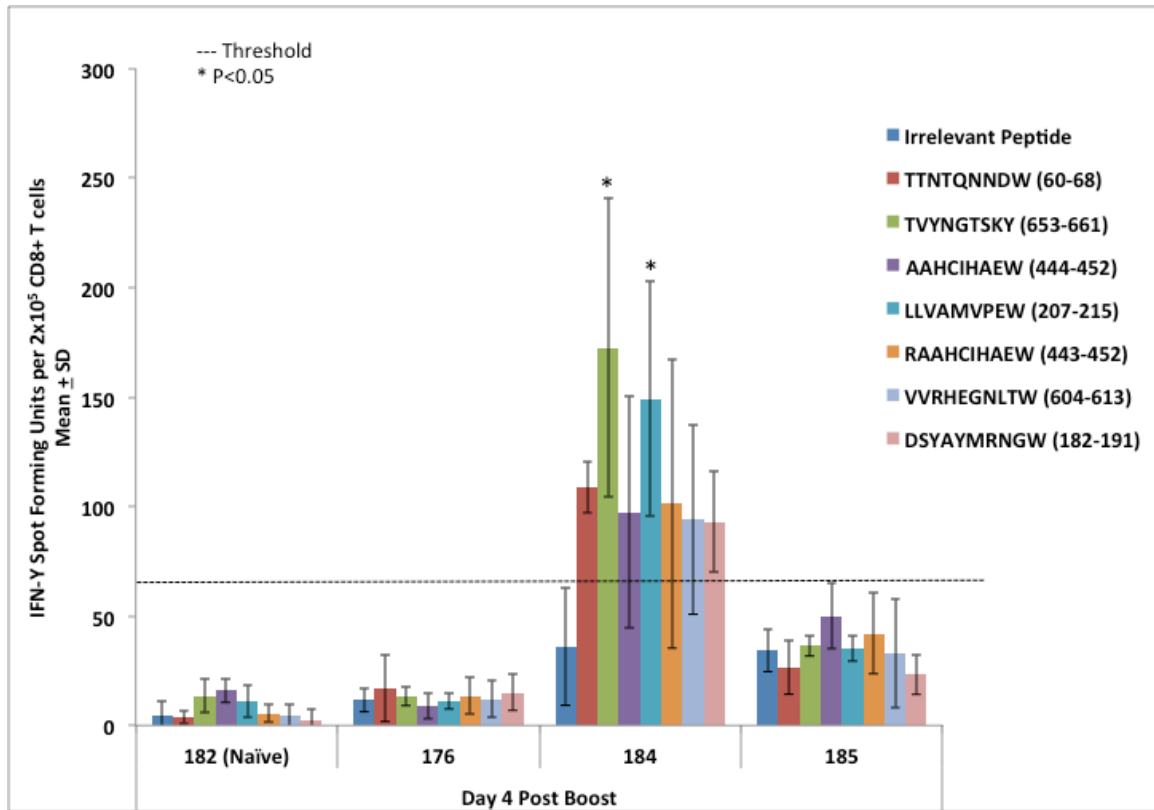


Figure 4-4: CD8 T cells from Ad5-P1-Ii two vaccinated animals (184 and 185) gave a slight response to two epitopes present in FMDV P1. CD8+ T cells from vaccinated cattle (176, 184, and 185) or naïve (182) were stimulated with FMDV peptides overnight and then tested by ELISpot assay for antigen-specific IFN- γ release. Values are means plus standard deviations (SD) (error bars) of four wells normalized to 2×10^5 CD8 T cells. Values which are statistically significantly different ($p < 0.05$) from the irrelevant peptide are indicated by asterisks. P-value = .001 and .007 for 653TVYNGTSKY215 and 207LLVAMVPEW215 respectively. Dotted line is the threshold.

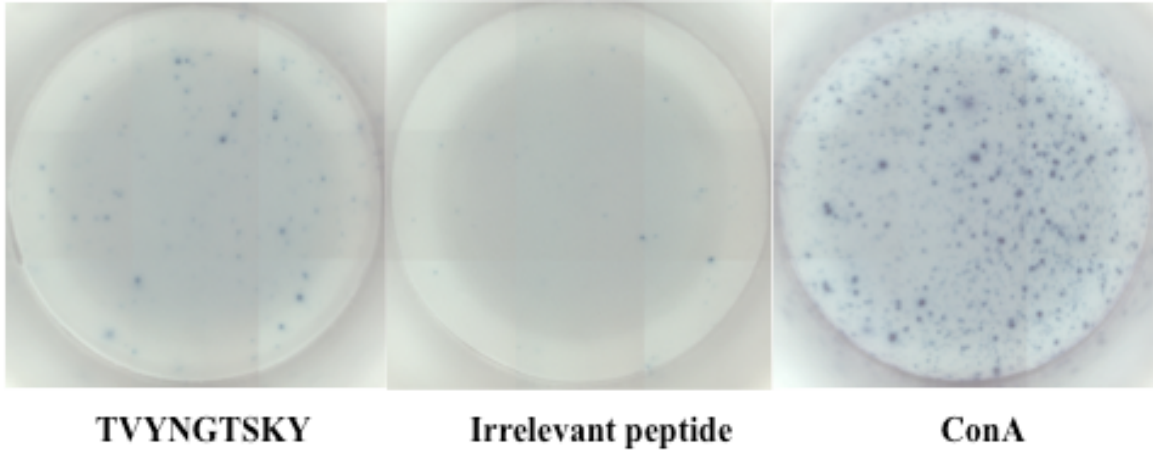


Figure 4-5: Representative images of the ELIspot wells. The wells are from steer 184 who was vaccinated with Ad5-P1-Ii. 653TVYNGTSKY215 (left image) shows a positive CD8+ T cell response as compared to spots induced by irrelevant peptide (center image) and ConA assay positive control is shown (right image).

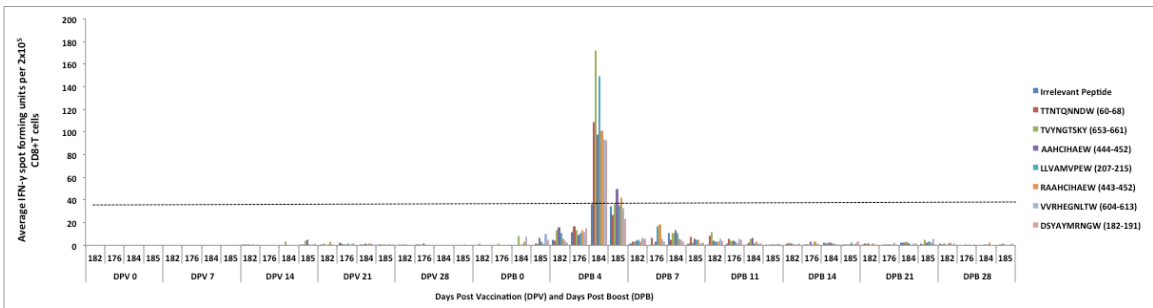


Figure 4-6: CD8 T cells from Ad5-P1-Ii vaccinated animals (176, 184 and 185) and naïve steer (182) only responded on day four boost in the IFN- γ ELIspot assay. All time points the assay was performed is shown. Values are means plus standard deviations (SD) (error bars) of four wells normalized to 2×10^5 CD8 T cells. Values which are statistically significantly different ($P < 0.05$) from the irrelevant peptide are indicated by asterisks. Dotted line is the threshold.

Table 4-2: Supplemental Table 1

Supplemental Table 1: FMDV-derived peptides across serotypes

Accession #	FMDV Serotype	Peptide Sequence
AAT01710.1	P1-A24	LLVAMVPEW
AAT01707.1	A22/IRAQ	LLVAMVPEW
AAT01743.1	Asia-1	****L***L
AAT01753.1	Type C	****L***M
AAT01758.1	Type O1/BFS	*****L
AAT01779.1	Type O/UK	*****L
AIT55230.1	SAT-1	VAMVPELCK
AAT01792.1	SAT-2	VAMVPELCS
AAT01796.1	SAT- 3	VAMVPELHS

Accession #	FMDV Serotype	Peptide Sequence
AAT01710.1	P1-A24	TVYNGTSKY
AAT01707.1	A22/IRAQ	*****
AAT01743.1	Asia-1	YNGKTAYGE
AAT01753.1	Type C	YTGTTYTS
AAT01758.1	Type O1/BFS	VYNGECRYS
AAT01779.1	Type O/UK	VYNGNCKYG
AIT55230.1	SAT-1	TA*NGDCK*
AAT01792.1	SAT-2	TV*NGECE*
AAT01796.1	SAT-3	YNGNCKYSE

Accession #	FMDV Serotype	Peptide Sequence
AAT01710.1	P1-A24	TTNTQNNDW
AAT01707.1	A22/IRAQ	*****
AAT01743.1	Asia-1	*N*****
AAT01753.1	Type C	*****
AAT01758.1	Type O1/BFS	*****
AAT01779.1	Type O/UK	*****
AIT55230.1	SAT-1	N*QNNDWFS
AAT01792.1	SAT-2	N*QNNDWFS
AAT01796.1	SAT-3	N*QNNDWFS

Accession #	FMDV Serotype	Peptide Sequence
AAT01710.1	P1-A24	AAHCIHAEW
AAT01707.1	A22/IRAQ	*****
AAT01743.1	Asia-1	HCIHSEWDT

AAT01753.1	Type C	HCIHAEWDT
AAT01758.1	Type O1/BFS	AHCIHAEWD
AAT01779.1	Type O/UK	AHCIHAEWD
AIT55230.1	SAT-1	SHCYHAEWD
AAT01792.1	SAT-2	****YHS**
AAT01796.1	SAT-3	HCYHSEWDT

Accession #	FMDV Serotype	Peptide Sequence
AAT01710.1	P1-A24	RAAHCIHAEW
AAT01707.1	A22/IRAQ	AAHCIHAEW
AAT01743.1	Asia-1	HCIHSEWDT
AAT01753.1	Type C	HCIHAEWDT
AAT01758.1	Type O1/BFS	AHCIHAEWD
AAT01779.1	Type O/UK	AHCIHAEWD
AIT55230.1	SAT-1	SHCYHAEWD
AAT01792.1	SAT-2	AAHCYHSEW
AAT01796.1	SAT-3	HCYHSEWDT

Accession #	FMDV Serotype	Peptide Sequence
AAT01710.1	P1-A24	VVRHEGNLTW
AAT01707.1	A22/IRAQ	QYSGTINLHF
AAT01743.1	Asia-1	YSGTMNVHFM
AAT01753.1	Type C	YAGTINLHFM
AAT01758.1	Type O1/BFS	QYSGTINLHF
AAT01779.1	Type O/UK	QYSGTINLHF
AIT55230.1	SAT-1	YSGTLNLHFM
AAT01792.1	SAT-2	SGSLNYHFMY
AAT01796.1	SAT-3	SGSINLHFMY

Accession #	FMDV Serotype	Peptide Sequence
AAT01710.1	P1-A24	DSYAYMRNGW
AAT01707.1	A22/IRAQ	*****
AAT01743.1	Asia-1	G*****
AAT01753.1	Type C	K*****
AAT01758.1	Type O1/BFS	*****
AAT01779.1	Type O/UK	*****
AIT55230.1	SAT-1	HSYIRNGWDV
AAT01792.1	SAT-2	YTYMRNGWDV
AAT01796.1	SAT-3	HAYVRNGWDV

Accession #	FMDV Serotype	Peptide Sequence
AAT01710.1	P1-A24	KVYANIAPTY
AAT01707.1	A22/IRAQ	*****H
AAT01743.1	Asia-1	***M*A****
AAT01753.1	Type C	*****N
AAT01758.1	Type O1/BFS	*****N
AAT01779.1	Type O/UK	*****N
AIT55230.1	SAT-1	VYV NI APT NV
AAT01792.1	SAT-2	VY ANI APT NV
AAT01796.1	SAT-3	Y ANI APT NVY

^aAmino acids which are identical to the amino acid in the FMDV A24 sequence are indicated by an asterisk.

Bolded amino acids indicate similarities with the A24 peptide

5. CHAPTER 5: CONCLUSION

5.1. Summary

It was hypothesized that increasing our knowledge on the BoLA class I diversity in Holstein cattle and together with the translation of current technologies to assess T cell responses would lead to a better understanding of the bovine adaptive immune response to FMDV. The objectives of this work were to quantify, define and develop methods to detect polymorphisms of the BoLA class I genes of Holstein cattle (Chapter 2), to define the FMDV capsid protein peptide repertoire bound by BoLA class I molecules (Chapter 3) and quantify and define the antigen specific response of Holstein cattle to FMDV vaccination (Chapter 4).

Despite the extensive undertaking to establish the assays and experimental approaches to study the BoLA class I diversity and assess the bovine adaptive immune response to FMDV, much remains to be understood. We have just begun to scratch the surface regarding the diversity in the BoLA class I region. As additional herds in the US and worldwide are rapidly and accurately BoLA genotyped using molecular methods, such as Illumina, novel alleles or variants of existing alleles will be identified and should be deposited into curated databases to further our knowledge on the depth of diversity in this region. As a result this can be a starting point to study the genetic basis for disease resistance. Very few studies in the literature and now the work presented herein, have studied the breed-specific host response to FMDV. Generation of *in silico* epitope predictions in combination with synthetic MHC molecules and vaccine constructs specifically designed to target the cellular immune response have changed the way we are

able to study the adaptive immune response of livestock. As part of this dissertation, new BoLA molecules were synthesized and their peptide binding profiles were analyzed. Next, I used the *NetMHCpan* algorithm to predict several peptides that would bind with strong affinity and stability to one of the most common alleles found in our herd (BoLA-2*00801). These peptide predictions, along with BoLA peptide binding matrix predictions (PSCPL), followed by *in vitro* biochemical affinity and stability assays, narrowed the list of potential FMDV peptide epitopes. These peptide epitopes were then tested in ELISpot and tetramer assays with cells from animals vaccinated with a T cell directed adenovirus vaccine (Ad5-P1-Ii). From this work, two novel FMDV T cell epitopes were identified and demonstrated a CD8⁺ T cell response measured by IFN- γ release after vaccination with Ad5-P1-Ii. Based on the methods and results described in my dissertation, additional future projects ideas could be undertaken, including those discussed below.

5.2. Further lines of inquiry arising from this work

5.2.1. Determine the role of adenovirus in bovine CD8 T cell responses

Previous studies in mice and non-human primates have shown that intramuscular immunization with replication-defective recombinant human adenovirus serotype 5 (rHuAd5) elicits a CD8⁺ T cell response with a extended contraction phase and sustained memory population (Bassett et al. 2011; Steffensen et al. 2013; Steffensen et al. 2012; Tatsis et al. 2007; Yang et al. 2007). The following questions arose after our vaccine trial experiment: Did the steers generate a specific T cell response to the human

adenovirus 5 vector? Are human and bovine adenoviruses cross-reactive? Did pre-existing vector immunity from the initial vaccination prevent adenovirus from inducing strong CD8⁺ T cell memory and recall responses following the boost? In order to address some of these questions, steers should either be boosted a second time or the initial experiment should be repeated with a prime/boost regimen with Ad5-P1-Ii (the construct used in the current analysis) or Ad5-EGFP (another construct developed in our laboratory) to investigate the phenotype and function of bovine CD8⁺ T cells induced by the adenoviral vectors. To characterize the memory phenotype, the CD8⁺ T cells should be stained with memory cell surface markers CD62L, CCR7 and CD127 (Thakur et al. 2012). Additionally, an IFN- γ ELISpot, similar to the assay outlined in my dissertation, should be performed to identify the function of these CD8⁺ T cells. Following vaccination, the frequencies of epitope-specific CD8⁺ T cells could be determined by either intracellular cytokine staining after incubation with adenovirus-specific peptides or with tetramers. To date, only eight CD8 T cell epitopes to adenovirus have been identified; the *NetMHCpan* algorithm could be used to discover additional adenovirus peptides (Leen et al. 2008). As shown in mice, preexisting immunity to adenoviral vectors (Ad5 and A35) does not interfere with eliciting a CD8 memory and recall response; it would be noteworthy to observe if the result is similar in bovine (Steffensen et al. 2012).

A total of 51 human adenovirus serotypes and 8 bovine adenovirus serotypes have been identified. It is possible the steers used in the Ad5-P1-Ii vaccine study were previously exposed to a bovine adenovirus that interfered with their response to human

Ad5 vector. However, this seems unlikely because phylogenetic analysis of the hexon protein (capsid protein) indicates human and bovine adenoviruses are quite divergent (Davison et al. 2003). Therefore, using the human Ad5 vector as a vaccine delivery system in cattle should not be a problem. Adenoviral vectored vaccines have been tested in dogs, cats, poultry and livestock (Ferreira et al. 2005). Similar to the Ad5-FMD vaccine, other Ad-vectored vaccines have been developed for rabies virus, feline immunodeficiency virus, avian infectious bronchitis virus, porcine respiratory and reproductive syndrome virus and bovine viral diarrhoea virus (Ferreira et al. 2005). Adenoviral vectors have been known to induce potent cell-mediated and humoral immune responses, making them ideal vaccine vectors against intracellular pathogens (Tatsis and Ertl 2004). However, more work needs to be conducted to better understand the immune response induced by these vectors in economically important species, such as cattle.

5.2.2. Establish a proteomic approach for epitope discovery

An alternative method for developing new vaccine candidates is to use immunoproteomics (the direct identification of BoLA class I presented epitopes) (Hoppe et al. 2010). Immunoproteomics starts with monitoring T cell reactivity upon transfection of antigen presenting cells with a pathogen gene of interest. After reactive T cells have been identified, smaller protein fragments can be de-convoluted and validated with mass spectrometry to reveal the epitope or epitopes. Immunoproteomics has been used to study several viruses, such as Influenza and Dengue virus, and to uncover MHC class I specific T cell epitopes naturally presented by infected cells (Testa et al. 2012a; Testa et al.

2012b). In order to apply this approach to cattle vaccinated or infected with FMDV, autologous fibroblasts or mouse P815 cells expressing individual BoLA class I genes infected with Ad5-P1-Ii or Ad5-Blue (empty vector) could be used. Fibroblasts generated from skin biopsies could be used for MHC class I antigen presentation. The protocol for the isolation and generation of these cells has been established in our laboratory, as well as others (Childerstone et al. 1999; Kandasamy et al. 2011). The peptide-MHC complexes presented on the surface of these cells could be isolated with immunoaffinity chromatography using a pan-specific MHC class I antibody (W6/32 or IL-A88) coated with protein A/G beads. The bound peptide-MHC complexes are eluted from the beads by adding acetic acid. The eluent is then heated to dissociate the bound peptides from the MHC molecules. The peptides are separated from the antibody by centrifugation, and the purified peptide mixture is then fractionated using high-performance liquid chromatography (HPLC) (Figure 5-1). Mass spectrometry is then carried out using Orbitrap. HPLC purified peptide fractions are then injected individually into the liquid chromatography-mass spectrometry (LC-MS/MS) system to identify the peptide sequence (Figure 5-1) (Testa et al. 2012a). The spectra data can be searched using SEQUEST search engine on Proteome Discoverer 1.4 (Thermo Fisher Scientific, Waltham, MA, USA) against a curated Bovine Uniprot database. The result files will be further analyzed by Scaffold 4.3 (Proteome Software, Portland, OR, USA) to compare the unique peptide counts and to identify gene ontology functions of the identified proteins.

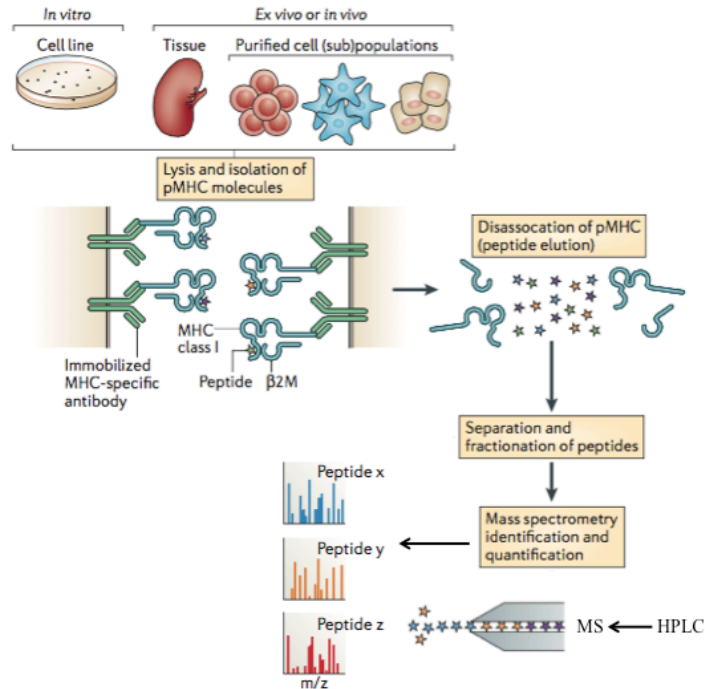


Figure 5-1: Peptide discovery workflow. Figure modified from (Tschärke et al. 2015).

Alternatively, another approach that could be used for epitope discovery is SILAC technology (stable isotope labeling by amino acids in cell culture). In this approach, cells with a phenotype of interest are cultured in medium supplemented with isotopically labeled amino acids. Reference cells grown in normal medium are used as a control. After elution, peptides pools from the reference and experimental cells are analyzed by LC-MS/MS and their spectra are compared (Hoppe et al. 2010).

There are several challenges to the proteomics approach for epitope discovery. The analytical challenge lies in discriminating between the peptides identified from infected cells and non-infected related peptides that are presented on the cell surface. This could be overcome by validating the peptides in cellular assays demonstrating no CD8+ T cell reactivity to non-infected cells. Another caveat of this proteomics approach is that

this method can identify peptide sequences that have a slightly altered sequence, but are recognized by the same TCR as the true T cell epitope (ie, mimotopes). Further functional analysis, including tetramer and intracellular cytokine staining involving the epitope in question would be needed to verify the true T cell epitope identified by mass spectrometry. Regardless of these challenges, proteomics provides an alternative method to epitope discovery for studies of cytotoxic T cell immunity, ultimately revolutionizing vaccine design.

5.2.3. Describe functional MHC clustering across BoLA and SLA molecules

As described earlier *MHCcluster* is a functional tool used to cluster MHC molecules based on their predicted peptide binding specificities. After generation of the BoLA *MHCcluster* tree (Chapter 2), one interesting question arose: Are the functional specificities of BoLA similar to those of other important agricultural species, such as swine? Figure 5-2 shows the functional specificities of swine leukocyte antigen (SLA) molecules (red), BoLA molecules that have not been synthesized (blue) and BoLA molecules that have been synthesized (green). The tree highlights the functional overlap between BoLA and SLA molecules.

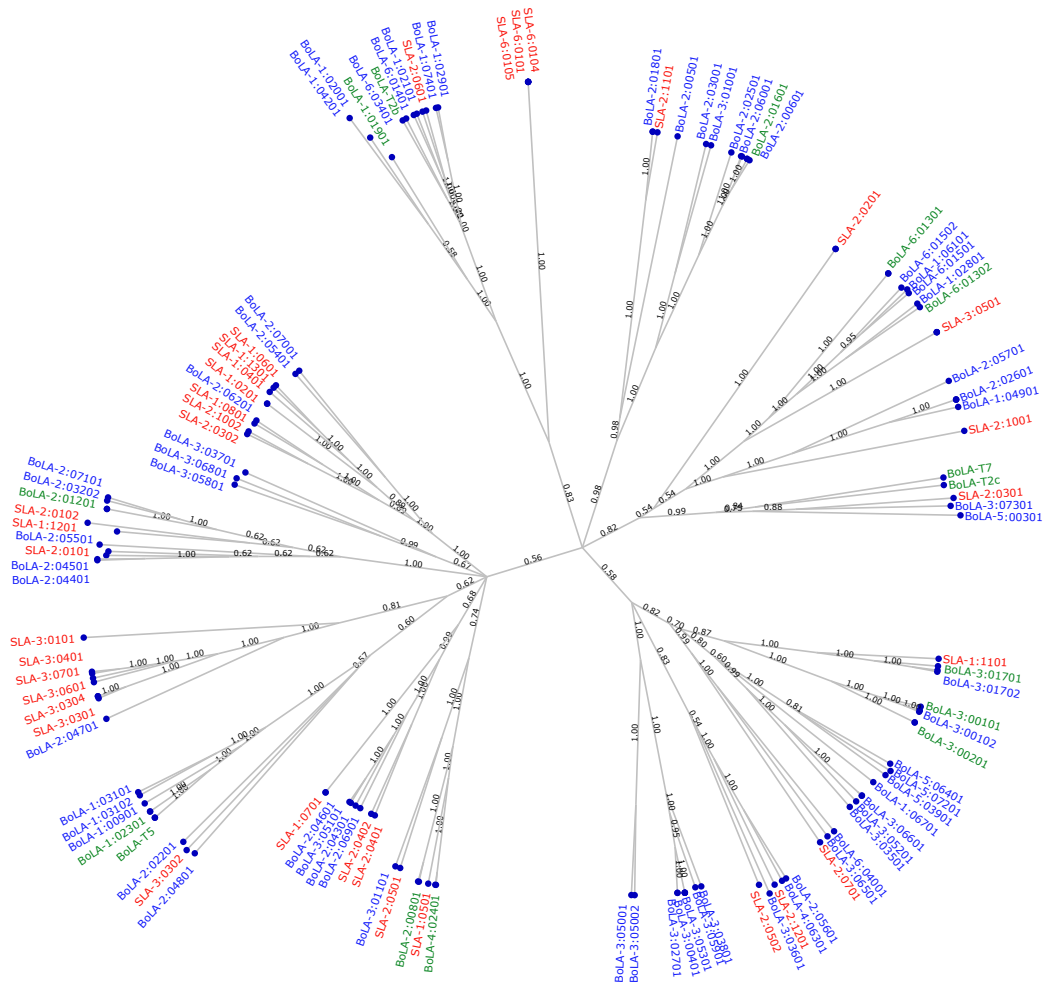


Figure 5-2: MHCcluster tree of BoLA and SLA molecules.

Additionally, Table 5-1 identifies similarities and differences in the key anchor positions of BoLA and SLA molecules that clustered together on the functional tree. For example, SLA and BoLA molecules appear to share amino acids Tyrosine, Phenylalanine, Tryptophan and Methionine in position nine. Position two is more variable, but common amino acids Valine, Threonine and Serine appear in both BoLA and SLA molecules. The shared overlap of the amino acids in these key anchor positions

could potentially help to identify epitopes that could be bound to both BoLA and SLA molecules. This tree, however, is a prediction of the functional specificities; whether or not the common SLA and BoLA alleles can actually recognize identical epitopes in context of vaccination or infection has yet to be determined. Additional biochemical affinity and stability data in swine and bovine are needed to validate the peptide binding motifs of these molecules. This data can then be used to iteratively train the *NetMHCpan* algorithm, strengthening future epitope predictions. If swine and bovine do have functional overlap, the concept of “supertypes” could be extended beyond just humans. Therefore, the ideal vaccine would be able to elicit an immune response in multiple species. This would be especially important for FMDV vaccine design, as FMDV is rampant in developing nations where multiple species are affected and resources are limited.

Many unanswered questions still remain. How important is the distribution of commonly expressed BoLA and SLA molecules in terms of protection or susceptibility from disease? How does the peptide-BoLA or peptide-SLA complex affect TCR activation? How does this correspond to the level of immunogenicity of the peptides? Is it possible to predict and monitor immune responses in outbred populations? How will these peptide epitopes influence vaccine design? The technologies developed in this dissertation can be used as the foundation to address some of these unanswered questions. Results generated from the proposed studies will provide knowledge on the mechanisms behind the immunological functions, which has implications for animal health.

Table 5-1: Key amino acids in anchor positions of SLA and BoLA molecules

	<u>Position 2</u>	<u>Position 3</u>	<u>Position 5</u>	<u>Position 7</u>	<u>Position 9</u>
SLA 1*0401	V,T,L,I	D,A,E			Y,F,W,M
BoLA 2*07001	T,S,V,A	A,D			Y,F,M,W
BoLA 2*05401	S, T	D,A,F			F,L,Y,I,W,V
BoLA 2*06201	S,L,T,V				Y,F,W
BoLA 3*03701	T,S,G,N	F,A,Y,I,V,M	F	A	F,L,Y,M,W
BoLA 3*06801	S,G,T,A	I,A	L		F,L,W,I,M
BoLA 3*05801	G,S,T,A	F,A			F,L,Y,W,M
SLA 2*0401					Y,F,W
SLA 2*0402		F,I,Y,A,V			Y,W,F,M
BoLA 2*06901	S,T,V,A	Y,F			Y,F,W,M
BoLA 2*04301					Y,F,W
BoLA 3*05101	G,S,T	F,V			Y,F,W,M
BoLA 2*04601	S,G,T	Y,F			Y,F,W,M
SLA 1*0701					Y,F

*Amino acids are represented by single letter codes

*Red highlights swine leukocyte antigen (SLA) molecules that have been synthesized

5.3. Final Thoughts

My research contributed to the field of veterinary immunology through the production of assays for characterizing the bovine immune response to intracellular pathogens, such as FMDV. Although many questions remain unanswered, ultimately, my research provided valuable tools for assessing the breed-specific host responses to pathogens by translating new technologies that have been applied in human vaccinology to the design of bovine vaccines. My research efforts were focused on advancing our understanding of BoLA class I antigen presentation and quantifying the antigen-specific response to FMDV to better understand adaptive immunity in Holstein cattle. I accomplished this by using cutting-edge technologies, including bioinformatics, high-throughput immunologic assays and development of adenoviral-vectored vaccines. The immune response is multifarious, and no branch of the immune system can work alone; rather, cell-mediated immunity must work in concert with humoral immunity to combat

the dynamic nature of pathogens. Remarkable progress has been made within the field of veterinary immunology and although much work remains, ultimately, this progress is leading to the advancement of cattle health.

5.4. Comprehensive Bibliography

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