CHARACTERIZATION OF *DEFB103* GENE STRUCTURE, GENE EXPRESSION AND ITS HAPLOTYPE ASSOCIATION WITH THREE COMMON DISEASES IN BEEF AND HOLSTEIN CATTLE

A Thesis Submitted to the
College of Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in the Department of Animal and Poultry Science
University of Saskatchewan
Saskatoon, SK
Canada

Ali Mirabzadeh-Ardakani, M.Sc.

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work, or in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying, publication, or use of this thesis or parts of it thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make use of material in this thesis in whole or in part should be addressed to:

Head of the Department of Animal and Poultry Science

University of Saskatchewan

Saskatoon, Saskatchewan, S7N 5A8

ABSTRACT

The *DEFB103* gene is a member of the β-defensin gene family, and it has not been well studied in cattle. In this study *DEFB103* gene expression, gene structure and its haplotypes association with three diseases were studied. *DEFB103* gene expression was profiled for 27 tissues in nine two-week old calves and the transcript was most abundant in tissues with stratified squamous epithelium. An age-dependent decrease (P <0.05) in *DEFB103* gene expression was observed in buccal epithelium when comparing healthy two-week old and 10- to 12-month-old calves. A bovine herpesvirus-1 respiratory infection did, however, significantly (P < 0.05) up-regulate *DEFB103* gene expression in the buccal epithelium of 6- to 8-month-old calves. Immunohistochemistry was used to identify cells expressing DEFB103 protein within tissues with stratified squamous epitheliums to confirm the *DEFB103* gene expression results. DEFB103 protein was most abundant in basal epithelial cells and was present in these cells before birth. A close association was observed between the dendritic cells and epithelial cells expressing DEFB103 in both the fetus and newborn calf, and this is consistent with the β-defensin regulatory effect on dendritic cell responses.

DEFB103 cDNA boundaries were determined using RT-PCR. A newly discovered non-coding exon 1a and a 261 bp intron 1a were identified in cattle. At least two complete copies of *DEFB103* with an ATG start codon are present in cDNA in some cattle. *DEFB103* sequence assemblies and partial cloning cloning sequences revealed two types of deletion (4-bp and 8-bp) in the 5′UTR.

Two novel SNPs were identified in the 5′UTR of the *DEFB103* (c.-383A>G, c.-241G>A) in addition to five (c.-319A>G, c.-264C>T, c.-69A>G, c.-42A>G, and c.-34G>A) previously

reported SNPs in the 5′UTR of *DEFB103* gene. The association among three diseases and four haploid haplotypes was studied. Diploid haplotype analysis shows a trend toward increased risk of mastitis (1/4; P= 0.053) in the Saskatchewan population and a decreased risk of mastitis (4/4; P= 0.06) in the Pennsylvania population. An inconsistent trend toward decreased (2; P=0.09) or increased (4; P=0.09) risk of mastitis also were determined when the haploid haplotypes were compared to others in the Saskatchewan and the Pennsylvania populations. There was no significant difference in the haplotype frequencies of the control group and cattle with Cancer-eye (P= 0.43). No significant difference was determined in the survival curve (P=0.50), the level of serum haptoglobin (P=0.65), or the level of interferon-gamma (P= 0.50) between cattle with diploid haplotypes 2/2 and 2/4 and shipping fever.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my supervisors Dr. Sheila Schmutz and Dr. Philip Griebel, for their countless hours of support, guidance, encouragement, and comments and editing expertise. Their profound understanding, valuable ideas, and professional advice led me through the difficult terrain of scientific research.

I would like to extend thanks to my graduate advisory committee, Dr. Bernard Laarveld (chair), Dr. Fiona Buchanan, Dr. Andrew Van Kessel and Dr. Vikram Misra, for the guidance, support, critiques, and encouragement every step of the way. A special thanks goes to my external examiner, Dr. Graham Scoles for his comments and discussion.

I would like to acknowledge and extend my gratitude for the financial support this project received from the Saskatchewan Agriculture Development Fund (20020270). Thanks to Dr. Gregory Penner for allowing me to collect tissue samples from the animals of his projects. I would like to thanks, Patricia Gonzalez-Cano, Jay Solie, Robert Brownlie, Ian Shirley and Rahwa Osman from Dr. Griebel's lab at VIDO.

I would like to thank my family: my mother, my father, my brother and my sister for supporting me spiritually all the way through my study and my life in general.

Last but not the least; I would like to thank my wife, Marziyeh Khani, from the bottom of my heart. I would not have been able to complete this program without her unconditional support and her endless patience.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

µl	Microlitre
°C	Degrees Celsius
BE	Basal epithelial
BHV-1	Bovine herpesvirus-1
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BTA	Bovine Chromosome
CBD103	*Canine DEFB103*
CD	Cluster of Differentiation
cDNA	Complementary deoxyribonucleic acid
CNV	Copy number variation
CNVRs	Copy number variation regions
DCs	Dendritic cells
DEFB103	*β-Defensin 103*
dH_2O	distilled H_2O
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate
DP	Dermal papillae
ESEs	Exon splicing enhancers
ESSs	Exon splicing silencers
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gDNA	Genomic DNA
GWAS	Genome-wide association study
hBDs	Human β-defensins
HFDs	High Fidelity Duplication
HSV	Herpes Simplex Virus

IFN-γ	Interferon-gamma
IGF-2	*Insulin-like growth factor 2*
IgG	Immunoglobulin G
IHC	Immunohistochemistry
ISEs	Intron splicing enhancers
ISSs	Intron splicing silencers
MC4R	*Melanocortin 4 receptor*
MgCl2	Magnesium Chloride
min	Minutes
MITF	*Microphthalmia Associated Transcription Factor*
Ml	Millilitre
mM	Millimolar
mm	Millimeter
mm^2	Square millimeter
mRNA	Messenger Ribonucleic Acid
NAHR	Non-allelic homologous recombination
ng	Nanogram
NHEJ	Non-homologous end joining
OR	Odds ratios
PBMC	peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR- Restriction Fragment Length Polymorphism
PMNs	polymorphonuclear cells
pmol	Picomole
qRT-PCR	Quantitative Real-Time PCR
RNA	Ribonucleic Acid
RR	Relative risk

RT-PCR	Reverse Transcription PCR
s	Seconds
S. aureus	*Staphylococcus aureus*
SEM	Standard Error of mean
SNP	Single Nucleotide Polymorphism
snRNPs	Small nuclear ribonucleoproteins
TAP	*Tracheal antimicrobial peptide*
TLRs	Toll-like receptors
u	Unit
UTR	Untranslated Region
VNTR	Variable number tandem repeats
β-Defensin	*Beta-Defensin*
µg	Microgram
µM	Micrometer

1.0 GENERAL INTRODUCTION

Both infectious and non-infectious diseases add significantly to livestock production costs. These costs may be related to either decreased growth or productivity, the cost of medical treatment, or the culling or death of animals. Genetic selection of animals has been a very effective strategy to eliminate single gene diseases, such as beta-mannosidosis (Leipprandt et al., 1999) in Salers cattle. However, the selection of animals for resistance to infectious disease has been much more challenging. Selecting animals that are less susceptible to disease would be one approach. Selection pressure on genes involved in the immune system, which is very complex, involving both innate and acquired immune responses (Mallard et al., 2014; Thompson-Crispi et al., 2014b), could be one strategy. However, genetic selection strategies have focused on both the innate and acquired immune systems in Holstein cattle (Thompson-Crispi et al., 2014c). Focusing on genes involved in the innate immune system may provide much broader disease protection since the innate immune system provides the first barrier of defense against infection. Furthermore, a number of innate immune genes, such as *TLR4* (Wang et al., 2007), have been implicated when analyzing single nucleotide polymorphisms in cohorts of disease resistant or susceptible food-producing animals.

The *defensin* gene family includes a large number of diverse genes that play multiple roles in innate immunity (Lehrer, 2004). The *β-Defensin* gene members have various antiviral and antibacterial activities and diverse gene expression patterns when comparing among different tissues. One member of the *β-Defensin* gene family is *β-Defensin 103* (*DEFB103*). *Defensins* have been widely studied in humans and gene copy number variation (CNV) of *DEFB103* has

been associated with specific diseases, such as psoriasis (Hollox et al., 2008) and Crohn's disease (Fellermann et al., 2006). Canine studies also reported an association between *DEFB103* and both coat color (Candille et al., 2007) and atopic dermatitis (Santoro et al., 2013).

The focus of this research was to further characterize the *DEFB103* gene in cattle. This research included analyzing the number of alleles and their haplotypes and investigating whether there were potential associations between *DEFB103* gene alleles and haplotypes with two infectious and one non-infectious disease. Bovine respiratory disease (shipping fever) and mastitis were used as examples of infectious disease, and ocular neoplasia (cancer eye) was used as a non-infectious chronic disease. The relevance of these disease studies was further evaluated by analyzing tissue-specific expression of the *DEFB103* gene and evaluating whether gender or infection altered *DEFB103* gene expression.

2.0 LITERATURE REVIEW

2.1 General Review of Gene Structure

The length of a gene in prokaryotes such as bacteria and some of the lower eukaryotes, such as yeast, is equivalent to the size of its protein product. In other words, 3N base pairs of DNA sequence encode a protein with N amino acids. Based on many studies of eukaryotic genes, such as ovalbumin and immunoglobulin, Gilbert (1978) reported that DNA coding sequences are not continuous. Gilbert (1978) introduced the concept of a 'silent' DNA sequence for the very first time; that interrupts coding sequences. The 'silent' DNA sequence became known later as introns. DNA sequences that code for a protein are called exons.

2.1.1 Promoter region

The promoter region is the DNA sequence that plays a regulatory role in transcription. The promoter sequence provides complementary sites for binding by RNA polymerase enzyme and transcription factors that are necessary for transcription (de Vooght et al., 2009). Mammals have two types of core promoter sequences, TATA box promoters and CpG island promoter motifs (Smale and Kadonaga, 2003; Carninci et al., 2006). The TATA box is usually 25 to 35 bp upstream of the start codon (ATG) of an exon, and it determines the direction of transcription (Smale and Kadonaga, 2003).

In CpG islands, cytosine nucleotides are linked to guanine nucleotides by a phosphate bond. The length of CpG islands is more than 200 bp (Gardiner-Garden and Frommer, 1987),

and about 48% of the potential promoter regions are within CpG islands (Suzuki et al., 2001). Gene transcription might be silenced if DNA methylation occurs in this area (Maston et al., 2006).

Other regulatory elements in the promoter region may also affect gene transcription. These elements are called either an enhancer or a silencer (Maston et al., 2006). Enhancers may be far from the transcription start site, about tens of kilobase pairs upstream of the core promoter, in an intron or even after the stop codon. Enhancers increase transcription, and their direction or distance relative to the transcription initiation site has no effect on their activity (Blackwood and Kadonaga, 1998; Maston et al., 2006). Silencers decrease the level of transcription, and most of their properties are similar to enhancers. Their sequence provides a binding site for negative transcription factors that are called repressors (Maston et al., 2006).

Mutations can occur in promoter elements, and they may change transcription factor binding sites. These kinds of changes could affect gene transcription and make an organism more susceptible to disease. It was reported that a 23 bp insertion-deletion polymorphism in the putative promoter region of the prion protein gene is associated with bovine spongiform encephalopathy (BSE), and individual cattle with an allele including a deletion were more susceptible to BSE (Sander et al., 2004; Haase et al., 2007).

Housekeeping genes such as *B-actin* and *GAPDH* are ubiquitously expressed, and they are necessary for the maintenance of cell function (Eisenberg and Levanon, 2013). On the other hand, some functional genes have a tissue-specific expression (Jacox et al., 2010) or are expressed during different developmental periods of an animal's life (Malmuthuge et al., 2012). Malmuthuge et al. (2012) reported that expression of Toll-like receptors was significantly lower in the healthy gastrointestinal tract of 6-month dairy calves in comparison to those that were only

3 weeks old. *Insulin-like growth factor 2* (*IGF2*) has four alternate promoters in cattle, and they generate seven transcripts. Liver is the only tissue with the *IGF2* expression for all three transcripts that are produced by the first promoter via RNA splicing (Goodall and Schmutz, 2007). Narciandi et al. (2011) also reported tissue-specific expression for the *β-Defensin* gene cluster on BTA13, in the reproductive tract.

2.1.2 RNA splicing

The transcription product in prokaryotes is called messenger RNA (mRNA), and it is ready for translation to protein. However, in eukaryotes, the transcription process generates precursor RNA (pre-RNA) that includes exons and introns. In eukaryotes, a couple of steps are needed to convert precursor RNA to messenger RNA (mRNA). In one of these steps, introns are excluded from precursor RNA and this process is called RNA splicing. RNA splicing occurs in the nucleus (Han et al., 2011). A complex, called the spliceosome, is necessary for splicing. It includes five uridine-rich small nuclear ribonucleoproteins (snRNPs) along with other specific proteins that are involved in binding to the snRNPs (De Conti et al., 2013).

These snRNPs recognize the exon-intron junction sequences. The U1 snRNP recognizes the AG/GU sequence at the 5′ (donor) splice site. A GU pair of nucleotides always occurs at the 5′ end of introns. An AG pair of nucleotides is prevalent at the 3′ end of exons (Figure 2.1). The U2 snRNP recognizes the branch point that is rich in pyrimidine. It is involved in a lariat formation that aids in excision of the intron. The U2 Auxiliary Factor (U2AF) recognizes AG/G sequence at the 3′ end of the intron, known as the acceptor splice site. AG always occurs at the 3′ end of introns, and G is the prevalent nucleotide that exists at the 5′ end of exons (Figure 2.1) (McManus and Graveley, 2011; De Conti et al., 2013). In addition to these sequences, other regulatory sequences were identified in intronic and exonic regions. They might be enhancer or

silencer elements. They are called, exon splicing enhancers (ESEs), exon splicing silencers (ESSs), and intron splicing enhancers (ISEs) and intron splicing silencers (ISSs) based on their position (McManus and Graveley, 2011).

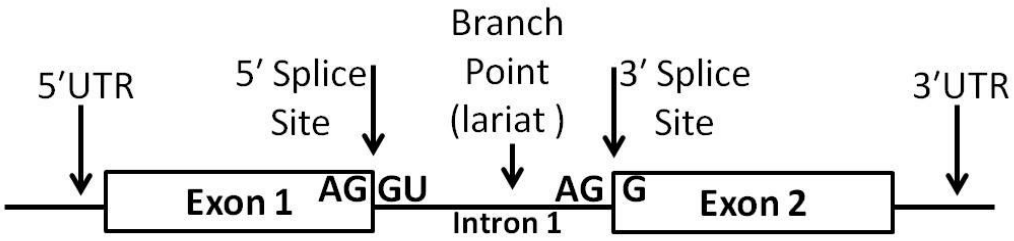


Figure 2.1: Basic concept of RNA splicing. A schematic picture of a pre-mRNA and the prevalent consensus sequences for the 5′ splice site, branch point, and 3′ splice site.

These enhancer and silencer signals in the exonic and intronic regions are one of the regulatory elements of alternative splicing (Goren et al., 2006). In alternative splicing, two or more mRNAs are generated from a single pre-RNA. Alternative splicing is usually tissue-specific, and it is critical in evolution and cell differentiation (McManus and Graveley, 2011). Genes with multiple promoters tend to have alternative splicing sites (Xin et al., 2008). *IGF2* and *microphthalmia-associated transcription factor (MITF)* are examples of such genes in cattle. Alternative splicing has been reported for *IGF2* in cattle (Goodall and Schmutz, 2007), pig (Amarger et al., 2002), sheep (Ohlsen et al., 1994), human (Holthuizen et al., 1993), rat (Holthuizen et al., 1993) and mouse (Holthuizen et al., 1993) (Figure 2.2).

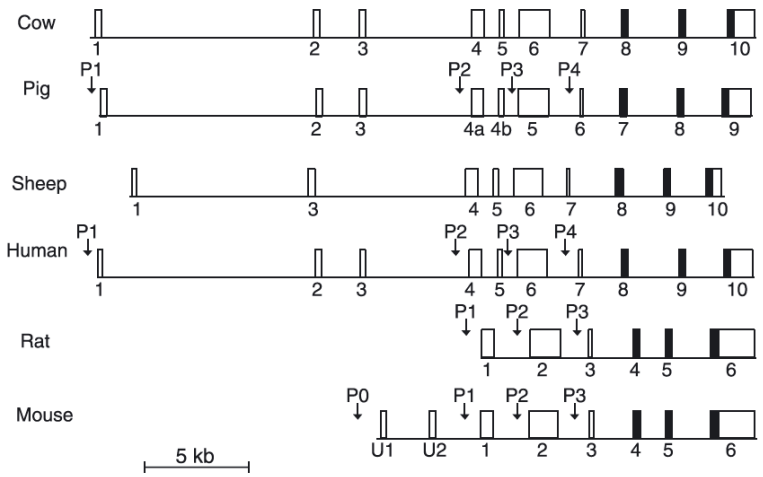


Figure 2.2: *IGF2* gene structure in different species. Promoters are called by P0–P4. Numbers identify *IGF2* exons. White boxes represent non-coding exons, and black boxes represent coding exons. U1 and U2 in Mouse *IGF2* indicate upstream exons 1 and 2 (Goodall and Schmutz, 2007), reprinted with permission from John Wiley and Sons License Number: 3851061009244.

2.1.3 Genotype and Haplotype

A variant of a gene is called an allele. This variation could be a single nucleotide polymorphism (SNP) (Figure 2.3 a), deletion or insertion (Alkan et al., 2011). Depending on the type of variation and its position in the coding sequence, it can have a different effect. A premature stop codon can be caused by a SNP or a frame shift in amino acid resulting from a deletion or insertion. This mutation is called a nonsense mutation (Flanigan et al., 2011). A missense mutation is a nucleotide substitution that causes a change in an amino acid in a protein (Buchanan et al., 2002). This change might be within a similar group of amino acids, and it is then called a conserved mutation. A non-conserved mutation is another type of missense mutation in which the new amino acid is from another group (i.e. acidic instead of basic). A

silent or synonymous mutation is a nucleotide substitution that causes no change in an amino acid sequence. It usually occurs in the third or wobble position of the codon (Komar, 2007).

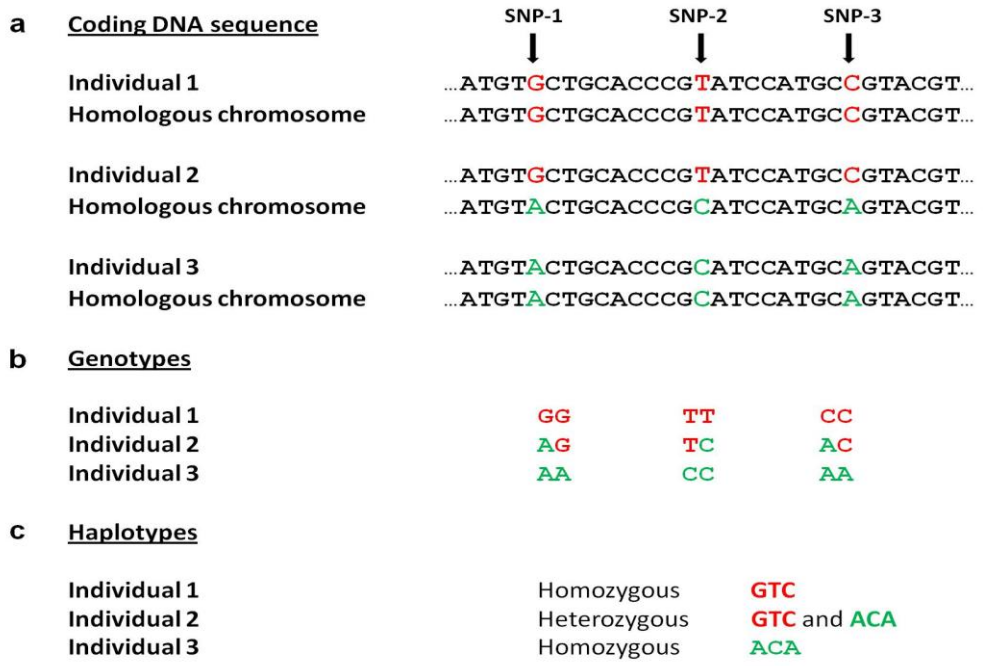


Figure 2.3: Coding DNA sequences with SNPs, Genotypes, and Haplotypes. **a)** A coding sequence of DNA from three individuals and their homologous chromosome that include three SNPs. **b)** Genotype is a combination of two alleles in a locus on homologous chromosomes. **c)** A haplotype is a combination of alleles from multiple SNPs on a single chromosome.

Animals have two sets of chromosomes (diploid) (Valero et al., 1992). Therefore, each animal carries two alleles of each gene, and this combination is called its genotype (Figure 2.3b). A haplotype is a combination of multiple alleles in a particular region of a chromosome (haploid) (HapMap, 2003). Each SNP has two alleles, but a particular region of a chromosome might have more than two haplotypes based on the various combinations of several genotypes (Figure 2.3c).

Haplotype data is often used in human population studies to find an association with a disease (Trowsdale and Knight, 2013). A genome-wide association study (GWAS) was

conducted by Abdel-Shafy et al. (2014) to test the effect of haplotypes on somatic cell score in German Holstein cattle. Ten Haplotypes with significant association (negative or positive) were identified in different genomic regions, such as *Bos taurus* autosome (BTA) 5, 6, 13, 18, 19 and X. Huang et al. (2014) studied haplotypes of the *IGF2* gene in Chinese Qinchuan cattle. They identified 4 SNPs and eight haplotypes among 723 individuals. The mutant haplotype, ATGG, showed a significant association with withers height (P = 0.004), and body weight (P = 0.018).

2.1.4 Gene orientation

The direction of a DNA strand is conventionally 5′-3′. The start codon is near the 5′ end and the stop codon is near the 3′ end of a gene in the forward orientation. Genes that are in the same direction with the DNA strand (5′-3′) are called forward, and those oriented in the opposite direction are called reverse (Figure 2.4). Davila Lopez et al. (2010) reported that about 50% of neighboring genes have the same orientation in humans.

The randomness of gene direction in different species was statistically tested by Li et al. (2012). This test indicated that gene orientation in at least one of the chromosomes of 99% of the tested species is non-random, and all of the chromosomes in archaea and bacteria have non-random orientation. Fifteen loci in cattle were tested for gene orientation randomness, and among these loci, three had non-random gene orientation.

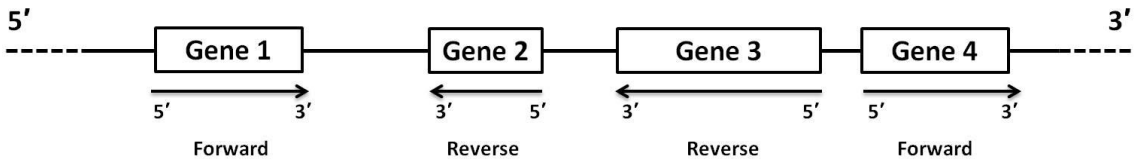


Figure 2.4: A schematic figure illustrating different gene orientation.

2.1.5 Pseudogenes

Pseudogenes do not code for a protein. A pseudogene is usually derived from a functional gene, sometimes called its "parent gene" (Mighell et al., 2000; Pink et al., 2011). Therefore, the pseudogene usually has high sequence homology to this "parent" copy, but the occurrence of a mutation, such as a premature stop codon or an insertion/deletion, makes the pseudogene unable to generate protein (Vanin, 1985; Balakirev and Ayala, 2003). Pseudogenes, like other non-coding DNA sequences, may have some regulatory role (Balakirev and Ayala, 2003).

There are two main types of pseudogenes: processed and unprocessed. A processed pseudogene is also called a retrotransposon. During the origin of processed pseudogenes, an mRNA transcript is reverse transcribed into DNA. This DNA is similar to complementary DNA (cDNA) and does not have introns or an upstream promoter sequence (Maestre et al., 1995). Duplication generates an unprocessed pseudogene. Cumulative mutations after duplication of a gene may interrupt gene function and thus create a pseudogene. Therefore, unprocessed pseudogenes have introns and upstream promoter sequence in their structure. An unprocessed pseudogene could be a complete copy or a partial copy of a gene (Rouchka and Cha, 2009).

2.2 Copy Number Variation (CNV)

Genomic variations occur in many different forms. Single nucleotide polymorphisms (SNPs) are substitution changes in DNA sequence. Variable number tandem repeats (VNTR) (Nakamura et al., 1987) are another form of variation that includes microsatellites and minisatellites. Microsatellites have 2 to 4 base-pair (bp) of DNA sequence that are repeated multiple times, and minisatellites have 10 to 100 bp of repeated DNA sequence (Ramel, 1997).

SNPs have been studied more than the other genetic variations. Nevertheless, development of sequencing techniques makes it possible for researchers to find microscopic and submicroscopic variations in a genome. These structural changes that include deletions, duplications, insertions, inversions, and translocations are called copy number variations (CNV) (Feuk et al., 2006; Zhang et al., 2009). Most CNVs are segments of DNA of about 1kb to 3 Mb. Whereas, structural variations that are smaller than 1kb are usually called small deletions or insertions (Feuk et al., 2006).

Redon et al. (2006) used SNP genotyping arrays, and Whole-Genome Tilepath BAC arrays to report a first-generation CNV map of the human genome. They indicated that CNVs covered approximately 12% of the human genome, and these sequence variations include a greater percentage of the human genome than SNPs. This percentage of CNV coverage is less than what Zhang et al. (2009) reported in their review (29.74%). Zhang et al. (2009) defined CNV as a DNA segment that is from 100bp to 3 Mb, whereas Redon et al. (2006) defined it as a DNA segment larger than 1kb, and also the resolution limitation of methods that were used for CNVs detection may cause overestimation.

Cattle CNVs were identified using array-CGH. In array-CGH, the fluorescence ratio of each chromosome is measured to determine locations of deletions and insertions in the test samples (Kallioniemi et al., 1992). The existence of cattle CNVs was confirmed by quantitative real-time PCR (qRT-PCR) for selected CNVs (Liu et al., 2008). Early estimates for CNV coverage in the cattle genome were 0.68% (22 megabases) (Fadista et al., 2010), and 1.07% (28.1 megabases) (Liu et al., 2010). Hou et al. (2011) used Bovine HapMap SNP genotyping data to estimate CNV regions (CNVRs) in cattle. They reported 682 candidate CNVRs that covered 4.6% (139.8 megabases). They reported that about 56% of the CNVRs were associated

with about 1263 genes that affect immunity, lactation, reproduction, and rumination. Most of the genes with CNV in cattle present in clusters, such as β –Defensins (Gallagher et al., 1995; Elsik et al., 2009).

Btau (Liu et al., 2009) and UMD (Zimin et al., 2009) are two available assemblies for cattle reference genome. Fine mapping of copy number variations using high-density BovineHD SNP array was performed on both assemblies by Hou et al. (2012). On Btau_4.0 about 3346 candidate CNVRs were identified that cover about 4.7% (142.7 megabases) of the cattle genome. Three thousand, four hundred and thirty eight CNVRs (146.9 megabases) were detected on the UMD3.1 assembly. The comparison of these two data sets indicate that UMD3.1 has 50% more and 20% longer CNVs. In UMD3.1, most of the unplaced contigs from sequencing were assembled, and it might be one of the reasons for this CNV abundance on the UMD3.1 assembly (Hou et al., 2012).

2.2.1 Mechanisms of CNV formation

CNVs can be inherited or generated during meiosis (Lupski and Stankiewicz, 2005). A recent study using HapMap and analysis of SNPs and CNVs indicated that about 80% of CNV differences are between individuals, and more than 99% of those were derived from Mendelian inheritance. Based on this information, *de novo* generation of CNVs is 100 times less than CNVs derived from inheritance (McCarroll et al., 2008). Copy number can be different in various tissues of an individual (Piotrowski et al., 2008). These findings indicate that CNVs can also be generated somatically (Hastings et al., 2009), and as a result, identical twins can have different sets of CNVs (Bruder et al., 2008).

Structural changes in a chromosome happen by homologous and non-homologous recombination (Hastings et al., 2009). Duplications, deletions, inversions, insertions and

combinations of rearrangements can lead to different copy numbers (Schaschl et al., 2009; Almal and Padh, 2012). Two primary mechanisms for CNV formation from these categories are non-allelic homologous recombination (NAHR) and non-homologous end joining (NHEJ) (Stankiewicz and Lupski, 2010).

2.2.2 Variation in copy number and disease susceptibility

CNV studies in different species revealed that the number of copies for a particular gene had an association with complex and common disorders. CNVs can have effects on disease in various ways such as gene dosage (Schaschl et al., 2009), gene interruption (Vincent et al., 2000), gene fusion (Woodwark and Bateman, 2011), and position effects (Henrichsen et al., 2009).

The peripheral myelin protein 22 (*PMP22*) gene expression is affected by gene dosage. *PMP22* is located in a 1.5 Mb duplication/deletion region on the short arm of chromosome 17 in humans. Charcot-Marie-Tooth peripheral neuropathy phenotype is linked to this area. Duplication of this locus causes *PMP22* overexpression and the manifestation of Charcot-Marie-Tooth disease in humans. On the other hand, deletion of this locus causes haplo insufficiency and *PMP22* under expression that increases hereditary neuropathy with liability to pressure palsies in humans. The deletion of 1.5 Mb includes 30 to 50 genes, but the *PMP22* gene is the only gene that shows a gene effect dosage (Lupski, 1998).

Another example of gene dosage is Parkinson's disease in humans. Parkinson's disease is one of the most common nervous disorders. It can affect approximately 1% of people over 50 years old (Polymeropoulos et al., 1996). Singleton et al. (2003) reported a triplication of the *SNCA* gene, located on 4q21, in a large family with autosomal dominant early-onset Parkinson's disease. The triplicated region size is about 2 Mb and includes the whole *SNCA* gene and 17 other

genes. Patients with *SNCA* duplication or triplication show different Parkinson's disease phenotypes. Duplication of *SNCA* causes classical Parkinson's disease while patients with the *SNCA* triplication have more severe phenotypes (Singleton et al., 2013). Therefore, *SNCA* copy number has a gene dosage effect on Parkinson's disease phenotypes.

Red-green color blindness in humans is an example of gene interruption. The red and green pigment genes have high sequence homology (98%) (Nathans et al., 1986). Therefore, they tend to have crossing over, and this can cause a series of duplications and deletions in these genes.

Many studies were conducted to show the *β-Defensins* CNV and their association with Crohn's disease in humans. Fellerman et al. (2006) reported that individuals with 3 or fewer copies of *DEFB4* (HBD2) are more susceptible to colonic Crohn's disease. Also, individuals with fewer copies of *DEFB4* had significantly ($P = 0.033$) lower mucosal mRNA expression. However, this association is in contradiction with the study by Bentley et al. (2010). They revealed that individuals with 4 or more copies of *DEFB4* were more susceptible to colonic Crohn's disease. One of the reasons for this contradiction might be their sample size. Fellerman et al. (2006) tested ten patients with Crohn's disease and ten controls. While, Bentley et al. (2010) examined 466 patients with Crohn's disease and 329 controls.

Unlike humans, fewer studies have been conducted in livestock to find an association between CNVs and diseases. Twenty CNVs were identified in cattle selected for resistance or susceptibility to intestinal nematodes (Liu et al., 2011). Liu et al. (2011) revealed that two main events occurred in a region that was related to ATP-binding cassette sub-family C member 4 (*ABCC4*), also known as the multidrug resistance-associated protein 4 (MRP4) gene. The expression of *ABCC4I* gene was up-regulated 14 days after *Cooperia oncophora* infection (Li and Gasbarre, 2009).

Hollox et al. (2008) suggested a model for the relation of CNV and the disease Succeptibility. “Increasing copy number increases susceptibility to inflammatory or autoimmune disease, but increases protection against infectious disease. Decreasing copy number increases susceptibility to infectious disease but decreases susceptibility to inflammatory or autoimmune disease”.

2.3 Defensins and Innate Immunity

Mammalian species are exposed to a wide variety of pathogenic and non-pathogenic micro-organisms. It is critical that host species discriminate between non-pathogenic micro-organisms, such as the commensal bacteria present on all body surfaces, and pathogenic micro-organisms that breach external barriers and establish an infection. Mammalian species have developed a variety of ways to protect themselves from these infectious agents, and collectively these protective mechanisms comprise the immune system. The immune system has been functionally divided into innate and adaptive immune responses (Kimbrell and Beutler, 2001) but it is now known that both arms of the immune system are functionally integrated and work in concert to prevent or clear microbial infections (Mantovani et al., 2011; Zak and Aderem, 2015).

All living organisms have an innate immune system (Kimbrell and Beutler, 2001) which is characterized by rapid activation within a short time following infection (Hazlett and Wu, 2011). The first line of defense in the innate immune system is a physical barrier, such as skin and mucosal surfaces. Chemical barriers such as antimicrobial peptides are produced by cells within the epithelial layer and provide a second line of defense. Antimicrobial peptides can act against a wide variety of pathogens (Agerberth and Gudmundsson, 2006). If, however, a pathogen evades this first layer of defense then innate immune cells can be recruited to control the pathogen replication. A wide variety of innate immune cells, including macrophages,

neutrophils, dendritic cells (DCs), and natural killer (NK) cells may be recruited. Antimicrobial peptides may play a role in the recruitment of these cells (Yang et al., 2002; Lai and Gallo, 2009) and cells, such as DCs then play a key role in linking the innate and adaptive immune system (Yang et al., 1999; Luster, 2002).

The adaptive immune response is present only in vertebrates (Pancer and Cooper, 2006). This system provides a response that is unique to individual proteins within pathogens and following initial exposure to a pathogen this first response may result in the activation of specific B cells and T cells. Furthermore, this activation of specific B and T cells can result in prolonged survival of these cells, which has been characterized as immune memory. This immune memory then facilitates a more rapid and stronger immune response following re-exposure to the same pathogen (Bonilla and Oettgen, 2010). This immune memory may then facilitate a much more rapid clearance of the pathogen following a secondary invasion or infection.

2.3.1 Antimicrobial peptides in innate immunity

Antimicrobial peptides are produced in mammals, insects, and plants and provide an important defense mechanism of the innate immune system (Lehrer and Ganz, 1992). These small polypeptides consist of fewer than 100 amino acids (Ganz, 2003a), and their production is highest in tissues and cells that provide a significant barrier to commensal and pathogenic microbes. Among these tissues, epithelial surfaces are the most important sites for the production of antimicrobial peptides in vertebrates, invertebrates, and plants (Ganz, 2003a; Ganz, 2003b). Defensins (Ganz et al., 1985; Selsted et al., 1985) and cathelicidins (Zanetti et al., 1995; Lehrer and Ganz, 2002) are the two major families of antimicrobial peptides in humans and other mammals. Defensins are highly expressed in mammalian epithelial tissues (Pazgier et al., 2006). Cathelicidins share some similarities with defensins in their sites of expression and their quantity

but they are structurally and evolutionary distinct (Zanetti et al., 1995; Lehrer and Ganz, 2002). Other antimicrobial peptides, including histatins (Tsai and Bobek, 1998) and Dermcidin (Schittek et al., 2001), are also present in mammals, but their presence is limited to specific animal species and tissues. Dermcidin expression was limited to sweat glands (Schittek et al., 2001). Expression of histatins was limited to the parotid and submandibular salivary glands in humans (Tsai and Bobek, 1998) and the old world Rhesus monkey (*Macaca mulatta*) but was not detected in mice (Dickinson et al., 1987).

2.3.2 Defensin gene family

Defensins are an important family, of antimicrobial peptides produced in both animals and plants (Thomma et al., 2002). Defensins are part of the innate host defenses that protect against infection by Gram-positive and Gram-negative bacteria, mycobacteria, fungi, and enveloped viruses when their concentration is 1-100 μg/ml (Ganz and Lehrer, 1995). Defensins are small arginine-rich cationic peptides (3-4 kDa). These small peptides, 16-50 amino acids, have a β-sheet rich folding (Ganz and Lehrer, 1995; Reddy et al., 2004; Jarczak et al., 2013). The first antimicrobial peptides identified with a beta-sheet structure were isolated from rabbit macrophages and antimicrobial peptides with a similar structure were then called defensins (Boman, 1995). This class of molecules is also characterized by three intramolecular disulfide bonds (six linked cysteines) (Reddy et al., 2004; Jarczak et al., 2013).

Peptide length, linking pattern of the conserved cysteine bonds, the site of protein production (Lehrer and Ganz, 1999; Ganz, 2003a), and tertiary structure (Ganz, 2003a; Selsted, 2004) are criteria used to separate defensins into α-, β- (Lehrer and Ganz, 1999) and θ-defensin (Tang et al., 1999; Selsted, 2004) subfamilies. It is argued that the α- and β-defensin families

arose from a common pre-mammalian gene based on their chromosomal location in humans (8p22-8p23), gene structure, and similar peptide precursors (Liu et al., 1997).

The α-defensins are also called "common classic defensins" with each member consisting of 29-35 amino acids (Jarczak et al., 2013). For α-defensins, the six conserved cysteines create three disulfide bonds between 1-6, 2-4, and 3-5 (Ganz, 2003a) and α-defensins are only expressed in mammals and marsupials. Therefore, they may have evolved before the divergence of placental mammals and marsupials (130 million years ago) (Lynn and Bradley, 2007). The presence of α-defensins has been reported in many mammalian species, including humans (Ganz and Lehrer, 1995), rabbits, guinea pigs (Jarczak et al., 2013), and the horse (Bruhn et al., 2007). It has been suggested that cattle (*Bos taurus*) do not express α-defensins. This conclusion is based on a search of GenBank sequences (Fjell et al., 2008; Zimin et al., 2009) and genome analysis (Lynn and Bradley, 2007) that did not reveal sequences homologous to human α-defensins. Cattle have a gene cluster containing 11 cathelicidins, but α-defensins genes are absent from this cluster. In contrast, humans and mice have multiple α-defensins but only a single cathelicidin gene (Zanetti, 2004).Therefore, cathelicidins may replace the function of α-defensins in cattle.

θ -Defensins are the most recent member of the defensin gene family. They contain 18 amino acids (Selsted, 2004). Six θ-defensin genes were reported in humans, chimpanzees, and gorillas, but all of them are pseudogenes because of the same premature stop codon mutation. However, functional θ-defensin genes were reported in some old world monkeys, such as *Hylobates syndactylus* (a lesser ape) and orangutans (Nguyen et al., 2003). The θ-defensins arose from the post-translational splicing of two α-defensin precursors and are only expressed in leukocytes of nonhuman primates (Tang et al., 1999; Nguyen et al., 2003; Selsted, 2004).

2.3.2.1 The β-Defensin subfamily

β -Defensins contain 38-50 amino acids (Jarczak et al., 2013). In the β –Defensin family, the six conserved cysteines create three disulfide bonds between cysteines 1-5, 2-4, and 3-6 (Ganz, 2003a). Based on phylogenetic analyses, β-defensins are older than α-defensins (Lehrer and Ganz, 1999). Previous studies have shown that most β-defensins are located within a single gene cluster. The location of the β-defensin gene cluster is on chromosome 8 in humans (Bevins et al., 1996; Pazgier et al., 2006) and on chromosome 16 in dogs (Patil et al., 2005). In cattle, three β-defensin clusters are present on chromosomes 27 (BTA27) (Gallagher et al., 1995) (Figure 2.5), BTA13 (Cormican et al., 2008) and BTA23 (Meade et al., 2014). Based on available data in GenBank, ten members of the β-defensin gene family are present on BTA27. The size of this gene cluster is about 1.3 Mbp (Figure 2.5). The β-defensin cluster on chromosome 27 shows copy number variation (Meade et al., 2014). There is another cluster of beta-defensins on BTA13 that contains 19 members (Cormican et al., 2008), and five members (DEFB110, DEFB111, DEFB112, DEFB113, and DEFB114) are on BTA23 (Meade et al., 2014). Genomic approaches and bioinformatic studies of the bovine genome created an opportunity to identify more β-Defensin genes in cattle. Cattle, with 57 β-Defensin genes, have the largest number of β-Defensin genes among vertebrates (Meade et al., 2014). As mentioned earlier, there are no α-Defensin genes in cattle (Fjell et al., 2008; Zimin et al., 2009). Therefore, the greater number of genes in the β-Defensin family may compensate for the lack of α-Defensins.

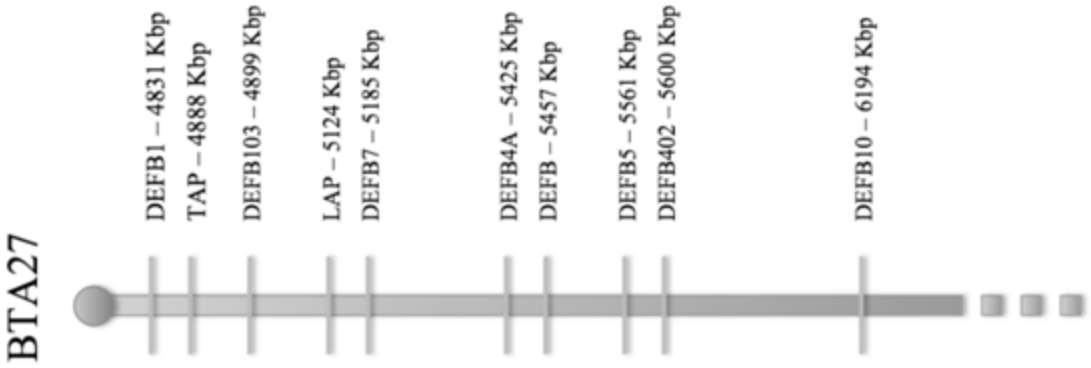


Figure 2.5: β-defensin gene cluster on chromosome 27 in cattle.

Tracheal antimicrobial peptide (TAP), the first defensin that was identified in cattle, has a broad spectrum of activity against fungi (*Candida albicans*), Gram-positive (*Staphylococcus aureus*), and Gram-negative (*Escherichia coli*) bacteria (Diamond et al., 1991). *TAP* is expressed in the respiratory mucosa in higher levels than lung tissue (Diamond et al., 1991), and its expression is up-regulated by bacterial infection (Diamond et al., 2000). TAP has been shown to have antimicrobial activity against *Staphylococcus aureus*, but *TAP* expression decreased when bovine mammary epithelial cells were infected with live *S. aureus* (Lopez-Meza et al., 2009). This observation may provide one explanation for how *S. aureus* evades innate immunity and remains the most common cause of chronic mastitis in cattle (Fox, 2012).

The next group of β-defensin peptides were isolated from cattle neutrophils (Selsted et al., 1993) and included DEFB4 and DEFB5 (Ryan et al., 1998). Ryan et al. (1998) reported that *DEFB4* and *DEFB5* were highly expressed in bovine alveolar macrophages. In contrast to *TAP*, *DEFB4* and *DEFB5* expression was up-regulated in both cistern and alveolar tissues of cattle mammary glands that were infected with *S. aureus* (Whelehan et al., 2011). This may reflect the recruitment of macrophages following bacterial infection.

The human *β-defensin 3* (*hBD-3*) or *DEFB103* is another member of the β-defensin family. Harder et al. (2001) isolated and characterized hBD-3 protein from human psoriatic

scales. The hBD-3 has antimicrobial activity against Gram-positive bacteria (*S. aureus*, multiresistant *S. aureus, and Streptococcus pyogenes*), Gram-negative bacteria (*Pseudomonas aeruginosa and E. coli*), and the yeast *Candida albicans* (Harder et al., 2001). Harder et al. (2001) observed the interaction of hBD-3 and *S. aureus* under transmission electron microscopy to understand how hBD-3 reacts to *S. aureus*. They realized that hBD-3 creates a perforation in the peptidoglycan cell wall of *S. aureus* in the exact same way that penicillin does as an antibiotic. This characteristic distinguishes hBD-3 from other defensins that create folded (mesosome-like) structure in a cell membrane (Shimoda et al., 1995).

Candille et al. (2007) reported that *CBD103* (*DEFB103* ortholog in dogs) binds to the *melanocortin 1 receptor* (*MC1R*) and effects pigment type-switching in domestic dogs and transgenic mice. They also reported that *CBD103* binds to *melanocortin 4 receptor* (*MC4R*) in mice and it causes feeding behavior suppression.

2.3.2.2 β-Defensin immunomodulatory activity

Linking innate and adaptive immune responses is critical for a coordinated host defense against infectious agents (Medzhitov and Janeway, 2000). In this situation, the innate immune system senses the danger of infection and informs the adaptive immune system to respond to this threat. The β-defensin antimicrobial peptides have a significant immunomodulatory effect, in addition to their direct antimicrobial role. A number of studies indicate that β-defensins play an important role in linking innate and adaptive immune responses (Yang et al., 1999; Hammad and Lambrecht, 2008).

Phagocytic cells, such as DCs (dendritic cells) and macrophages, can present antigen to T lymphocytes and induce an antigen-specific response (Sallusto and Lanzavecchia, 2002; Kabelitz and Medzhitov, 2007). Immature DCs and memory T cells express the CCR6 chemokine

receptor, which binds human β-defensins (hBDs). Therefore, hBDs can be chemotactic for these cells and recruit them to sites of infection (Yang et al., 1999). The hBD3 also bind to periodontal pathogens like *P. gingivalis* hemagglutinin B. This can reduce the cytokine stimulation because pathogen does not attach to the keratinocytes or DCs. This can improve the adaptive response and control the inflammation in the oral cavity (Diamond and Ryan, 2011). Garcia et al. (2001) reported that hBDs could function as a chemoattractant for monocytes but the specific receptor involved is not known.

Toll-like receptors (TLRs) are a well-characterized family of innate immune receptors expressed on a broad range of innate immune cells. TLRs play an important role in regulating both the innate and adaptive immune responses through activation and maturation of dendritic cells (Kabelitz and Medzhitov, 2007). Funderburg et al. (2007) reported that hBD-3 may be able to link the innate and adaptive immune system through TLR signaling. Recombinant hBD-3 was used to demonstrate a direct interaction with TLR1 and 2. The expression of a cluster of differentiation genes, such as CD80, CD86, and CD40, was increased following stimulation of myeloid DCs and monocytes with recombinant hBD-3.

3.0 TISSUE AND AGE-DEPENDENT EXPRESSION OF THE BOVINE *DEFB103* GENE AND PROTEIN[1,2]

3.1 Abstract

Beta-defensin 103 (DEFB103) shares little homology with nine other members of the bovine beta-defensin family on BTA27, and in other species DEFB103 protein has diverse functions, including antimicrobial activity, a chemoattractant for dendritic cells, enhancing epithelial wound repair and regulating hair color. Expression of the bovine *DEFB103* gene was surveyed in 27 tissues and transcript was most abundant in tissues with stratified squamous epithelium. Oral cavity epithelial tissues and nictitating membrane consistently expressed high levels of *DEFB103* gene transcript. An age-dependent decrease ($P < 0.05$) in *DEFB103* gene expression was only observed in buccal epithelium when comparing healthy 10- to 14-day-old and 10- to 12-month-old calves. A bovine herpesvirus-1 respiratory infection did, however, significantly ($P < 0.05$) up-regulate *DEFB103* gene expression in the buccal epithelium of 6- to 8-month-old calves. Finally, *DEFB103* transcript was low in lymph nodes draining the skin and at the limit of detection in other internal organs such as lung, intestine, and kidney. Affinity-purified rabbit antisera to bovine DEFB103 was used to identify cells expressing DEFB103 protein within

[1] A version of this chapter has been published: Mirabzadeh-Ardakani, A., Solie, J., Gonzalez-Cano, P., Schmutz, S.M. and Griebel, P.J. Tissue- and age-dependent expression of the bovine DEFB103 gene and protein. *Cell Tissue Res* **363** (2016), pp. 479-90.

[2] The immunohistochemistry section in this chapter was conducted by Jay Solie, a summer student in the lab of Philip Griebel.

tissues with stratified squamous epitheliums. DEFB103 protein was most abundant in basal epithelial cells and was present in these cells prior to birth. Beta-defensins have been identified as regulators of dendritic cell (DC) chemokine responses, and we observed a close association between DCs and epithelial cells expressing DEFB103 in both the fetus and newborn calf. In conclusion, bovine *DEFB103* gene expression is most abundant in stratified squamous epithelium with DEFB103 protein localized to basal epithelial cells. These observations are consistent with proposed roles for DEFB103 in DC recruitment and repair of stratified squamous epithelium.

3.2 Introduction

Antimicrobial peptides, also known as host defense peptides, are an important component of the innate immune system (Cederlund et al., 2011). They include several families of structurally diverse molecules, consisting of less than 100 amino acids, and perform diverse functions, including both direct antimicrobial activity and modulation of innate immune functions (Ganz and Lehrer, 1994). The expression of antimicrobial peptides is highest in tissues and cells that provide an important barrier to commensal and pathogenic microbes, and this expression pattern is conserved among vertebrates, invertebrates, and plants (Ganz, 2003b; Ganz, 2003a).

Defensins are one family of antimicrobial peptides produced in mammals that share structurally similar motifs (Boman, 1995). They are 3- to 4-kDa cationic proteins with six conserved cysteine residues that create three cysteine–disulfide bonds (Ganz and Lehrer, 1994). Three different patterns of disulfide bonding among the conserved cysteines divides the defensin family into α-, β-, and θ-defensin subfamilies (Ganz and Lehrer, 1994; Lehrer and Ganz, 1999; Tang et al., 1999). The three cysteine–disulfide bonds of α-defensins are created by connecting

C1 to C6, C2 to C4, and C3 to C5 (Ouellette and Selsted, 1996), and cysteine cross-linking in β-defensins is C1 to C5, C2 to C4, and C3 to C6 (Tang and Selsted, 1993). In contrast, cyclic cysteine–disulfide bonds are created in the θ-defensin subfamily by connecting C1 to C6, C2 to C5, and C3 to C4 (Tang et al., 1999).

Based on phylogenetic analyses, β-defensins are older than α-defensins, and θ-defensins arose from two mutants of α-defensin genes in primates (Tang et al., 1999). A number of β-defensin genes are expressed in mammals, birds, and reptiles (Lehrer, 2004; Patil et al., 2004; Bagnicka et al., 2010) and most β-defensins occur within a single gene cluster. The β-defensin gene cluster is located on human chromosome 8 (Bevins et al., 1996; Pazgier et al., 2006), dog chromosome 16 (Patil et al., 2005), and cattle chromosomes 27 and 13 (Gallagher et al., 1995; Cormican et al., 2008). The genomic structure of most of the β-defensins consists of two exons and one intron. *DEFB105* has three exons and two introns (Pazgier et al., 2006), and a new non-coding exon has been reported in the 5′UTR of bovine *β-defensin 103* (*DEFB103*) and also predicted in ovine and caprine *DEFB103* (Mirabzadeh-Ardakani et al., 2014a). DEFB103 has the lowest amino acid homology when comparing the structure of mature peptides within the bovine β-defensin family (Figure 3.1a), and there is a relatively low level of homology when comparing DEFB103 among different species (Figure 3.1b). Therefore, this β-defensin may possess biological activities distinct from other β-defensin family members both within and among the different species.

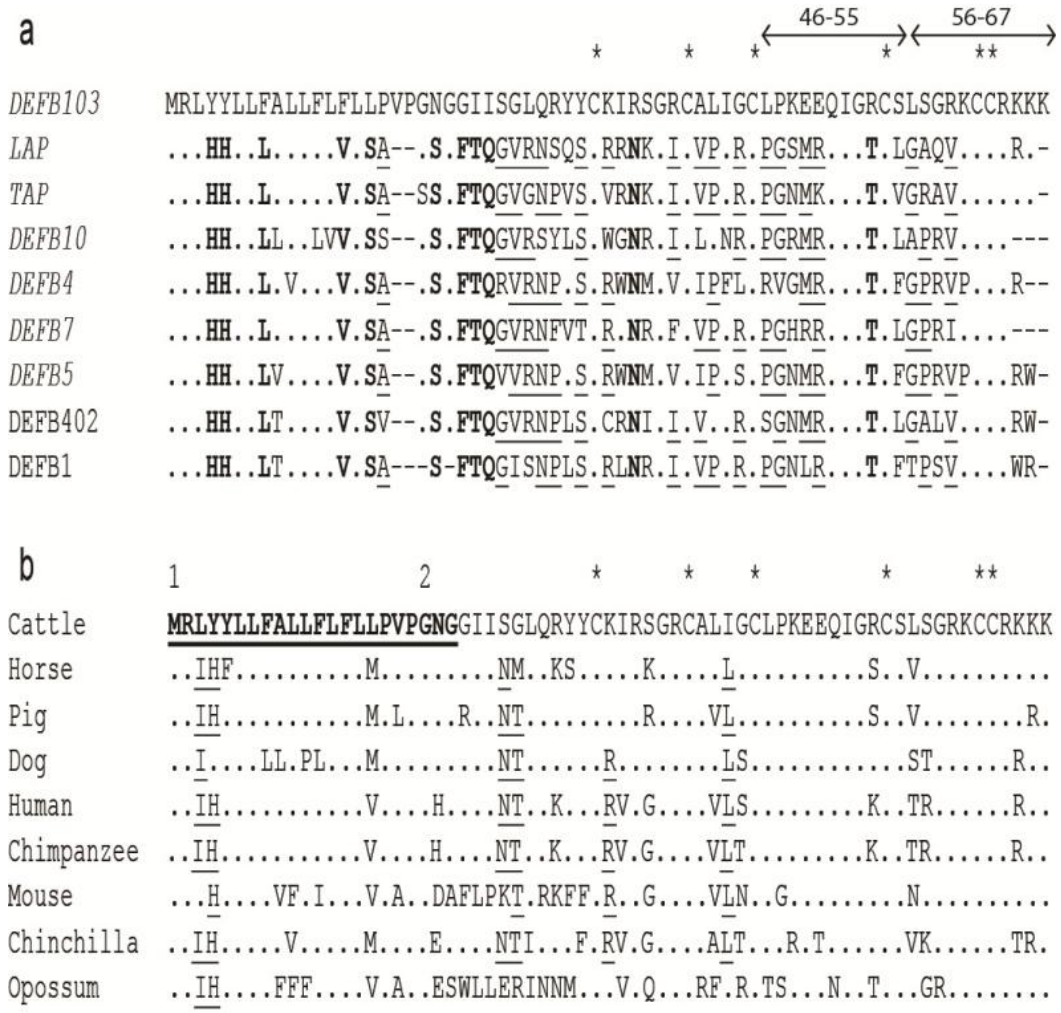


Figure 3.1: Amino acid alignment for β-defensins. a) Amino acid alignment for the β-defensin gene family on cattle BTA27. *Asterisks* indicate the six conserved cysteine amino acids. *Bold letters* indicate that all β-defensins have the same amino acid in the indicated position except DEFB103. *Underlined letters* show that 5 or more members of β-defensin gene family share the same amino acid in the indicated position, but it is not similar to DEFB103. *Arrows* indicate the two epitopes chosen to raise antisera for immunohistochemistry. b) DEFB103 amino acid alignment among different species. The first 22 amino acids (*bold and underlined*) in DEFB103 indicate the signal peptide. 1 and 2 show the start of the two coding exons. *Underlined letters* show that 5 or more members of the β-defensin gene family share the same amino acid in that position, but it is not similar to bovine DEFB103.

DEFB103 gene copy number variation has previously been reported with 2-12 gene copies per diploid genome identified in human (Hollox et al., 2003) and 2-4 gene copies per diploid genome reported for dogs (Leonard et al., 2012). A higher genomic copy number of β-defensin genes has been linked to a greater susceptibility to psoriasis (Hollox et al., 2008), and individuals with a lower human β-defensin-2 gene copy number also have a reduced transcript abundance (Fellermann et al., 2006). The bovine *DEFB103* gene also occurs in multiple copies in at least some individuals, based on sequence results from a cloning experiment (GenBank KM347983, KM347984, KM347985, and KM347986), and Reverse Transcription PCR (RT-PCR) data (Mirabzadeh-Ardakani et al., 2014a) but nothing is known regarding bovine *DEFB103* gene expression in specific tissues or whether expression is developmentally regulated or altered during an infection. Many epithelial tissues, particularly skin, express β-defensins (Pazgier et al., 2006; Candille et al., 2007; Leonard et al., 2012). Canine *DEFB103* gene (CBD103) expression was greatest in the tongue and was the most abundant β-defensin gene transcript detected in the skin (Leonard et al., 2012). Human *DEFB103* gene expression was detected in a wide variety of tissues, including oral epithelium, lung, trachea, skin, and neutrophils (Pazgier et al., 2006). Cross-reactive antisera to human β-defensin 103 (hBD-3) revealed detectable levels of CBD103 protein within epithelial cells located in the canine skin and around hair follicles (Leonard et al., 2012). In the present investigation, we hypothesised that bovine *DEFB103* gene expression was also restricted primarily to tissues containing epithelial cells. Real-Time Quantitative Reverse Transcription PCR (Real-Time qRT-PCR) was used to complete a broad survey of bovine tissues, and this analysis confirmed that tissues with stratified squamous epithelium had a 100- to 1000-fold higher level of *DEFB103* transcript. We then investigated whether expression of the *DEFB103* gene in epithelial tissues was altered by age, sex, or a viral respiratory infection.

Finally, immunohistochemistry (IHC) was used to determine if the production of DEFB103 protein was restricted to a particular subpopulation of the epithelial cell. This analysis confirmed that DEFB103 protein was produced primarily in basal epithelial cells and revealed a close association between dendritic cells and cells expressing DEFB103.

3.3 Materials and Methods

3.3.1 Tissue collection for RNA extraction

All experimental protocols for animals were reviewed and approved by the University of Saskatchewan Committee on Animal Care following guidelines approved by the Canadian Council on Animal Care. Calves were euthanized with an intravenous injection of 20 mL/45 kg Euthanyl (Bimeda-MTC, Canada) and all tissues for RNA extraction were collected within 15–30 minutes post-mortem. Tissues sampled included skin (lateral aspect of shoulder, axillary region, and perianal), blood (both peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMNs) were isolated separately (Whale et al., 2006)), tongue epithelium, buccal epithelium, tracheal mucosa, lung parenchyma, rumen, jejunum, ileum, caecum, rectum, testes, nictitating membrane (third eyelid), liver, spleen, kidney, bladder, adrenal gland, superficial cervical lymph node, skeletal muscle (shoulder), cardiac muscle, abomasal muscle, perirenal fat, and spinal cord. Tissues were collected from healthy 10- to 14-day-old male Holstein calves (n=9). To determine whether gender or a respiratory infection altered *DEFB103* gene expression tissue samples were obtained from the tongue and buccal epithelium of five male and five female 6- to 8-month-old Angus-cross beef calves 14 days after an experimental challenge with bovine herpesvirus-1 (BHV-1). A group of four castrated male calves, between 10 and 12 months of age, were used as uninfected controls for the collection of

the tongue and buccal epithelium. Calves between 6 and 12 months of age represent sexually immature animals. Details of the BHV-1 challenge study have been published by Griebel et al. (2014) and tissues were collected on day 14 post-BHV-1 infection. At this time, BHV-1 infection is being cleared (Griebel et al., 2014) and innate immune defenses, especially those involved in the repair of epithelium, may be activated. To determine whether age altered *DEFB103* gene expression, tissue samples were collected from the tongue, buccal epithelium, skin (shoulder), and rumen of four healthy, castrated male 10- to 12-month-old Holsteins. For the analysis of *DEFB103* gene expression, several 2-3 mm^2 pieces of tissue were immersed in 1 ml RNA*later*® (Applied Biosystem-Ambion) and kept overnight at room temperature before storing samples at −80 C until RNA was extracted.

3.3.2 Tissue collection for Immunohistochemistry

Approximately 5 mm^2 sections of nictitating membrane, buccal epithelium, rumen, shoulder skin, and bladder were collected following euthanasia of five 10- to 14-day-old, male Holstein calves and three fetal calves collected by caesarean section during the last trimester of gestation. Tissue samples were placed with the epithelial surface supported by a 3- to 4-mm-thick section of fresh liver placed on a strip of acetate film. Tissue blocks were slowly immersed in liquid N_2 to snap-freeze and then stored in sealed cryovials at −80°C.

3.3.3 RNA extraction

Total RNA was extracted from tissues stabilized by RNA*later*® using the TRIzol method (Invitrogen). Tissue fragments were homogenized in TRIzol solution by using a bead beater. Extracted RNA was treated with DNase-I (RNase-free; Invitrogen) to remove DNA, and RNA quantity and quality were then determined using a NanoDrop 2000 (NanoDrop Technologies,

DE, USA) spectrophotometer following the first extraction and an Agilent 2100 Bioanalyzer (G2938B; Agilent Technologies, Mississauga, Ontario, Canada) following the second RNA extraction. RNA with an RNA integrity number higher than 7 was considered acceptable for cDNA preparation.

3.3.4 Real-Time qRT-PCR primer design and validation

A primer pair for *DEFB103* (GenBank EU715240.1) was designed using the Clone Manager 9.0 program (Sci-Ed Software): forward primer 5'-CTTGTTCTTGCTGCCTGTTC-3' in exon 1; reverse primer 5'-GCCTATCTGTTCCTCCTTTG-3' in exon 2. A single copy of the *DEFB103* gene was cloned into a plasmid to determine the amplification efficiency of the primer pair. The linear range of amplification was determined by using serial dilutions of plasmid between $3 \text{ X } 10^0$ to $3 \text{ X } 10^5$ molecules. A standard curve (Appendix A) was drawn, and a linear regression equation was determined using Excel. Exponential amplification (Eq. 3.1) and efficiency percentage (Eq. 3.2) (Nolan et al., 2006) were calculated to evaluate amplification efficiency.

$$\text{Exponential amplification} = \left[10^{(-1/\text{Slope})}\right] \qquad \text{(Eq. 3.1)}$$

$$\text{Efficiency } (\%) = \left[\left[10^{(-1/\text{Slope})}\right] - 1\right] \times 100 \qquad \text{(Eq. 3.2)}$$

The calculated exponential amplification for the *DEFB103* primer pair was 2.03, with a calculated efficiency of 103.5%. *B-actin* (GenBank AF191490) was used as a housekeeping gene and the primer pair used (forward primer 5'-AGGCATCCTGACCCTCAAGTA-3'; reverse primer 5'-GCTCGTTGTAGAAGGTGTGGT-3') was previously validated for both specificity and amplification efficiency (Charavaryamath et al., 2011). *B-actin* efficiency was 101% and it was similar to the *DEFB103* primer pair efficiency.

3.3.5 cDNA synthesis and Real-Time qRT-PCR of cattle DEFB103

Total RNA (250 ng) was reverse-transcribed using qScript™ cDNA SuperMix (Quanta Biosciences™), following the manufacturer's protocol. The cDNA was diluted 1:10 using UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen) and then used as a template for the Real-Time qRT-PCR. Real-Time qRT-PCR was performed using Perfecta™ SYBR® Green SuperMix for iQ™ (Quanta Biosciences), following the manufacturer's protocol. The primer pair was used to amplify a 124-bp fragment of *DEFB103* that included part of both *DEFB103* coding exons and flanked the intervening intron. A single product was amplified, and the product was sequenced to confirm identity with *DEFB103*.

3.3.6 Generation of DEFB103 Antisera and Immunohistochemical staining of tissues

To validate the Real-Time qRT-PCR analysis of *DEFB103* gene expression in newborn calves and identify cells producing DEFB103 protein, a rabbit polyclonal antisera was commercially produced by Pierce Custom Services (Thermo Fisher Scientific). Two epitopes were designed and synthesized (Pierce Custom Services) to include amino acids 46-55 (LPKEEQIGRC) and 56-67 (SLSGRKCCRKKK) located near the C-terminus of the mature bovine DEFB103 protein (Figure 3.1a). The criteria used to select these two epitopes were predicted immunogenicity (Pierce Custom Services) and a comparison of amino acid sequences among known bovine β-defensins on BTA27. This comparison was completed using sequences with following accession numbers: DEFB103 (NP_001108334.1); LAP (NP_982259.3); TAP (NP_777201.1); DEFB10 (NP_001108556.1); DEFB4 (NP_777200.1); DEFB7 (NP_001095832.1); DEFB5 (NP_001124233.1); DEFB402 (CAD91246.1); and DEFB1 (NP_783634.1) (Figure 3.1a). Conservation of the bovine DEFB103 amino acid sequence among

species was also analyzed using the following accession numbers: cattle DEFB103; ACH69778.1; horse DEFB103; CAJ01801.1; pig DEFB3; NP_999609.1; dog CBD103; AAY59710.1; human HBD3: NP_061131.1; chimpanzee DEFB103; NP_001123222.1; mouse DEFB14: AAI16200.1; chinchilla DEFB1: AAM97293.1; opossum DEFB103: XP_001381623.1 (Figure 3.1b).

To generate specific antisera to bovine DEFB103, two rabbits were immunized with either peptide 46–55 or peptide 56–67 (Figure 3.1a) conjugated to KLH. The rabbits were bled monthly, and antibodies specific for each peptide were detected by ELISA, using the 46-55 and 56-67 peptides to capture antibodies. Rabbit immunizations were repeated over a 6-month period until a high titre of antibody specific to the 46-55 peptide was consistently detected. ELISA never detected specific antibodies to the 56-67 peptide. Immunoglobulin G (IgG) was then purified by ammonium sulfate precipitation from the serum of the rabbit with the highest antibody titre to the 46-55 peptide. Purified IgG was then passed through an affinity column with conjugated 46-55 peptide (Pierce Custom Services). Specificity of the eluted IgG was again confirmed by ELISA, using the 46-55 peptide to capture IgG. This procedure generated affinity-purified IgG with a 46-55 peptide-specific titre exceeding 10,000 (inverse of IgG dilution giving a positive reaction in the ELISA). This affinity-purified rabbit IgG was then used for the bovine DEFB103 IHC studies.

Cryosectioning of frozen tissues and staining of cryosections were performed as described previously (Griebel et al., 1992). IHC staining with the affinity purified DEFB103-46-55 rabbit antisera was optimized using a Vectastain Elite ABC kit (Pierce) to visualize antibody binding. The rabbit anti-bovine DEFB103 antisera was titrated to identify the highest dilution (0.07 μg IgG/ml) that gave visible staining and an affinity-purified rabbit antisera to an irrelevant protein

(human CCR5; Sigma Aldrich) was used at the same concentration to control for non-specific antibody binding. The anti-CD205 monoclonal antibody was previously optimized for IHC staining of bovine tissues (Fries et al., 2011) and validated for reactivity with bovine DCs (Gonzalez-Cano et al., 2014).

3.3.7 Statistical analysis

All Real-Time qRT-PCR reactions were repeated in duplicate for each sample, and average values used for subsequent analyses. Tissues expressing *DEFB103* with cycle threshold (Ct) below 35 were considered positive and included in subsequent analyses. A one-way ANOVA with Tukey's post hoc analysis was used to determine which tissues had significantly different transcript abundance when comparing samples from either 10- to 14-day-old Holstein calves (n=9) or 6- to 8-month-old Angus calves following BHV-1 respiratory infection and to examine the effects of gender and infection. Student's t-test was used to analyse the effect of age on *DEFB103* transcript abundance within individual tissues.

3.4 Results

3.4.1 Comparison of DEFB103 gene expression in tissues collected from newborn calves

To determine whether *β-actin* was an appropriate house-keeping gene for the comparison of *DEFB103* transcript abundance in multiple tissues and animals, we first compared Ct values for *β-actin* among all tissues surveyed. This comparison revealed consistent *β-actin* expression levels in all tissues (mean $\pm$ SEM = 21.1 + 0.16) with the exception of rumen (mean $\pm$ SEM = 20.5 + 0.13) where *β-actin* transcript was significantly ($P < 0.05$) more abundant. The difference in *β-actin* transcript abundance in the rumen was less than one Ct, which could, however, result

in an underestimation of *DEFB103* transcript abundance in the rumen. Consistent *β-actin* transcript abundance in all other tissues, regardless of age, gender or viral infection provided evidence for consistent RNA quality and validated the use of ΔCt values when comparing *DEFB103* transcript abundance.

The comparison of *DEFB103* transcript abundance among all tissues surveyed in 10- to 14-day-old Holstein calves revealed buccal epithelium had a significantly ($P < 0.05$) higher expression level than all other tissues surveyed (Figure 3.2). Other tissues with stratified squamous epithelium, including tongue, nictitating membrane, and skin, from a variety of body sites, had the next high level of *DEFB103* expression and shared a similar level of transcript abundance (Figure 3.2a). Trachea, with pseudostratified epithelium, had the next highest level of *DEFB103* transcript abundance that was similar to lymph nodes draining the skin (Figure 3.2a). The rumen, which also contains stratified squamous epithelium, had over 100-fold less *DEFB103* transcript than buccal epithelium (Figure 3.2a). Furthermore, a wide variety of other tissues, including the gastrointestinal tract, other internal organs, and blood had detectable DEFB103 transcript levels that were over 1000-fold less than buccal epithelium (Figure 3.2b). Thus, buccal epithelium appeared to be unique in its level of *DEFB103* gene expression.

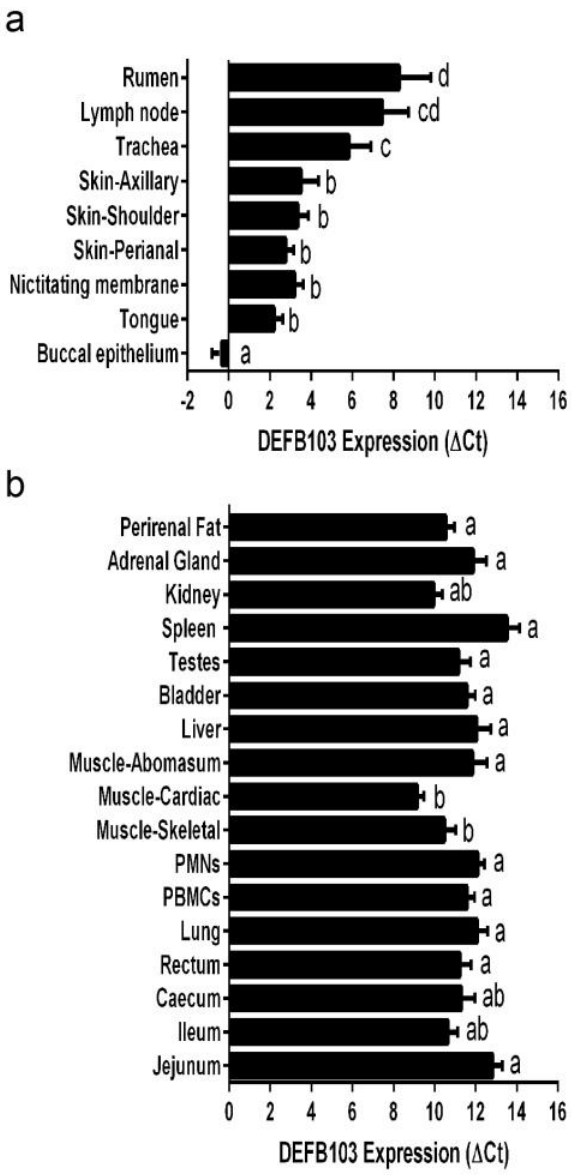


Figure 3.2: *DEFB103* gene expression in tissues collected from healthy 10- to 14-day-old calves. Expression of the *DEFB103* gene was analyzed by Real-Time qRT-PCR and is expressed as change in threshold cycle (ΔCt) relative to *β-actin*. Lower ΔCt values represent increased transcript abundance. RNA was isolated from 27 tissues (9 shown in a and 18 in b) collected from 10- to 14-day-old calves (n = 9). Each set of tissues was analyzed separately. Data presented are the mean + SEM and bars within each panel with different letters are significantly different (P <0.05).

3.4.2 Effect of age on DEFB103 gene expression

Tissues with stratified squamous epithelium had the most abundant *DEFB103* transcript in 10- to 14-day-old calves. These tissues were then collected from 10- to 12-month-old calves to determine if there was an age-dependent change in *DEFB103* expression levels. Similar levels of *DEFB103* expression were observed in skin, rumen, and tongue of newborn and older calves but there was a significant ($P < 0.05$) decrease in *DEFB103* transcript abundance in the buccal epithelium of older animals (Figure 3.3). Thus, buccal epithelium also appeared to be unique in that age influenced the level of *DEFB103* expression.

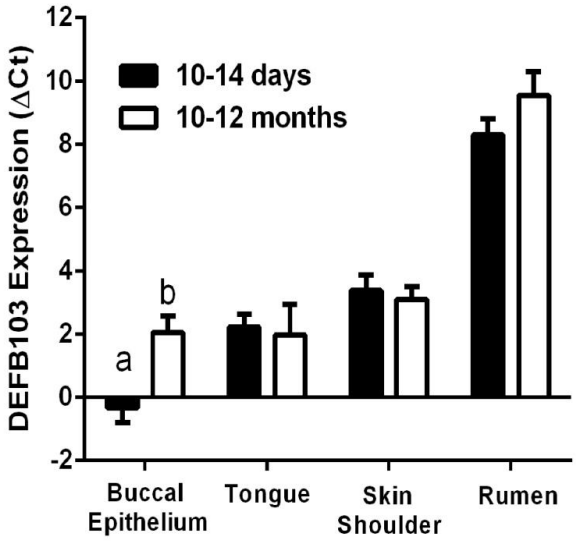


Figure 3.3: Age-dependent changes in *DEFB103* gene expression. *DEFB103* gene expression was compared to tissues with a high level of expression in newborn calves (10-14 days; $n = 9$) with similar tissues collected from adult (10-12 months; $n = 4$) animals. *DEFB103* gene expression was quantified by Real-Time qRT-PCR and expressed as a change in threshold cycle (ΔCt) relative to β-actin. Lower ΔCt values represent increased transcript abundance. Data presented are the mean + SEM and bars with different letters are significantly different ($P < 0.05$).

3.4.3 Effect of gender and viral infection on DEFB103 gene expression

Gender has been associated with significant differences in the expression of a wide variety of innate immune-related genes (Seillet et al., 2012). Therefore, we compared *DEFB103* transcript abundance in tongue, and buccal epithelium collected from 6- to 8-month-old female and castrated male calves, following an upper respiratory tract infection by BHV-1. No significant (P=0.09) gender difference was observed for either of these tissues (Figure 3.4a), and data were then combined to determine if a BHV-1 infection in the upper respiratory tract altered *DEFB103* gene expression. *DEFB103* transcript abundance remained unchanged in tongue epithelium when comparing tissues collected from uninfected and infected calves. In contrast, *DEFB103* expression was significantly ($P < 0.05$) up-regulated in the buccal epithelium of infected 6- to 8-monthold-old calves when compared to healthy 10- to 12-month-old calves (Figure 3.4b). Thus, buccal epithelium appears to be a site where both age and viral infection can modulate *DEFB103* expression.

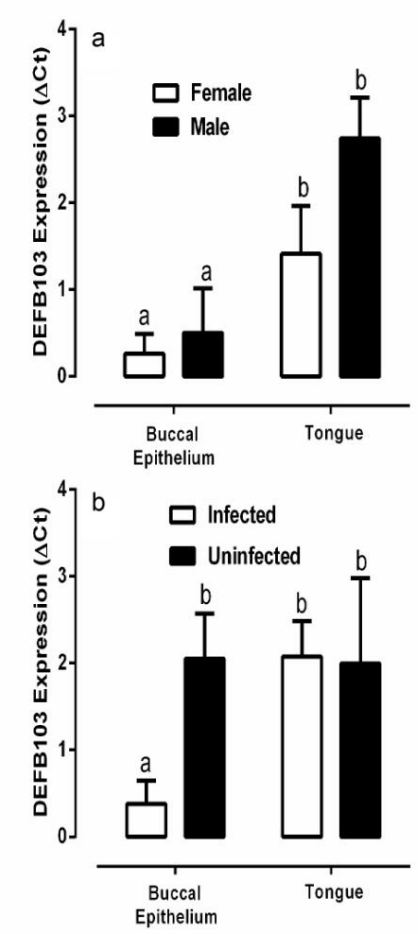


Figure 3.4: The effect of gender and viral infection on *DEFB103* gene expression in the upper respiratory tract. **a** *DEFB103* gene expression was compared in tissues collected from the oral cavity of 6- to 8- month-old infected male (*n*=5) and female (*n*=5) calves on day 14 following an experimental BHV-1 infection. **b** *DEFB103* gene expression was compared in tissues collected from the oral cavity of BHV-1 infected (*n* = 10) and uninfected (*n* = 4) calves. *DEFB103* gene expression was quantified by Real-Time qRT-PCR and expressed as a change in threshold cycle (*Δ*Ct) relative to β-actin. Lower *Δ*Ct values represent increased transcript abundance. Data presented are the mean + SEM and bars with different letters are significantly different (*P* <0.05).

3.4.4 DEFB103 protein expression in epithelial tissues

Transcript abundance may not correspond directly with protein abundance due to rapid mRNA degradation, restricted mRNA translation, or differences in protein turnover time (Boisvert et al., 2012). Therefore, immunohistochemistry (IHC) was used to confirm that tissues with stratified squamous epithelium, where the *DEFB103* transcript was most abundant, also expressed a high level of DEFB103 protein and to identify cells within epithelial tissues that produced DEFB103 protein.

Specificity of the affinity-purified rabbit antisera raised to the 46–55 epitope in bovine DEFB103 was confirmed in several ways. First, amino acid sequence alignment for DEFB103 and other known bovine beta-defensins determined that only three amino acids in peptide 46–55 (QIG) and peptide 56–67 (CCR) were common among all bovine β-defensins (Figure 3.1a). This level of conservation was considered insufficient to result in cross-reactive antisera (Pierce Custom Services). A concentration-matched, irrelevant affinity-purified rabbit antisera was also used to control for non-specific binding of rabbit IgG in tissue sections. No visible staining was observed with the irrelevant rabbit IgG in buccal epithelium collected from fetal and newborn calves (Figure 3.5a; Negative Control i and iv). Finally, bladder that had a very low level of *DEFB103* transcript (Figure 3.2b) was not visibly stained by the affinity-purified rabbit anti-bovine DEFB103 (Figure 3.6a). In contrast, tissue sections of buccal epithelium stained with affinity-purified rabbit anti-DEFB103 displayed intense staining localized to the basal epithelial cell layer but no visible staining of the dermis (DP) and superficial layers of the epidermis (Figure 3.5a; Bovine DEFB103 ii and v). While visible DEFB103 staining was restricted primarily to the single layer of basal epithelial (BE) cells in 10- to 14-day-old calves, there was

visible staining of both basal epithelial cells and several layers of epithelial cells superficial to the basal epithelium within the fetal buccal epithelium. (Figure 3.5a ii and v; Table 3.1).

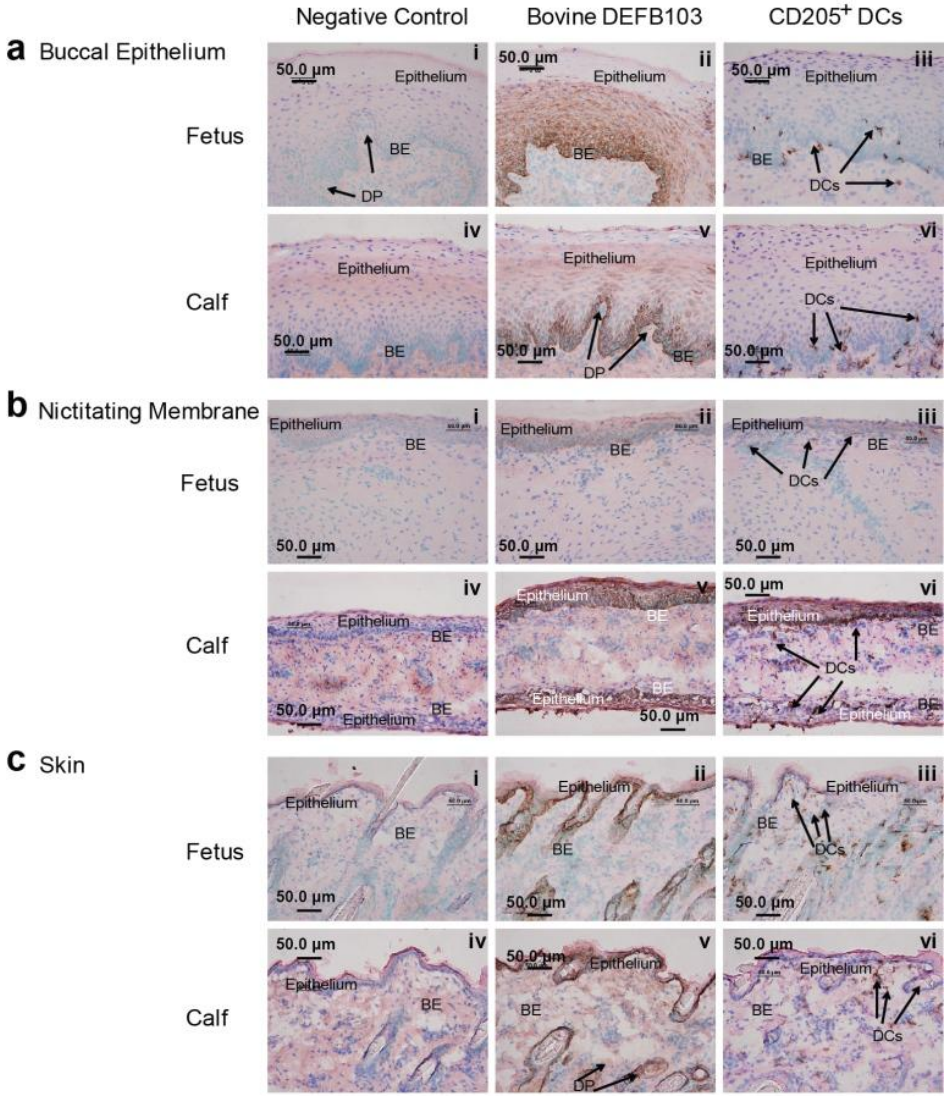


Figure 3.5: Immunohistochemical staining of buccal epithelium (**a**), bovine nictitating membrane (third eyelid) (**b**) and skin (**c**) with an irrelevant affinity-purified rabbit antisera (anti-human CCR5 protein; *i* and *iv*), affinity purified rabbit anti-bovine DEFB103 (*ii* and *v*) and monoclonal antibody specific to CD205 protein expressed on dendritic cells (DCs) (*iii* and *vi*). Staining presented is representative of that observed for tissues collected from fetuses (*i*, *ii*, and *iii*) and 10- to 14-day-old calves (*iv*, *v*, and *vi*). *BE* basal epithelial cell layer at the junction between the epidermis and dermis; *DP* dermal papillae are dermal projections into the epithelial cell layer of the skin. Scale bars 50 µM

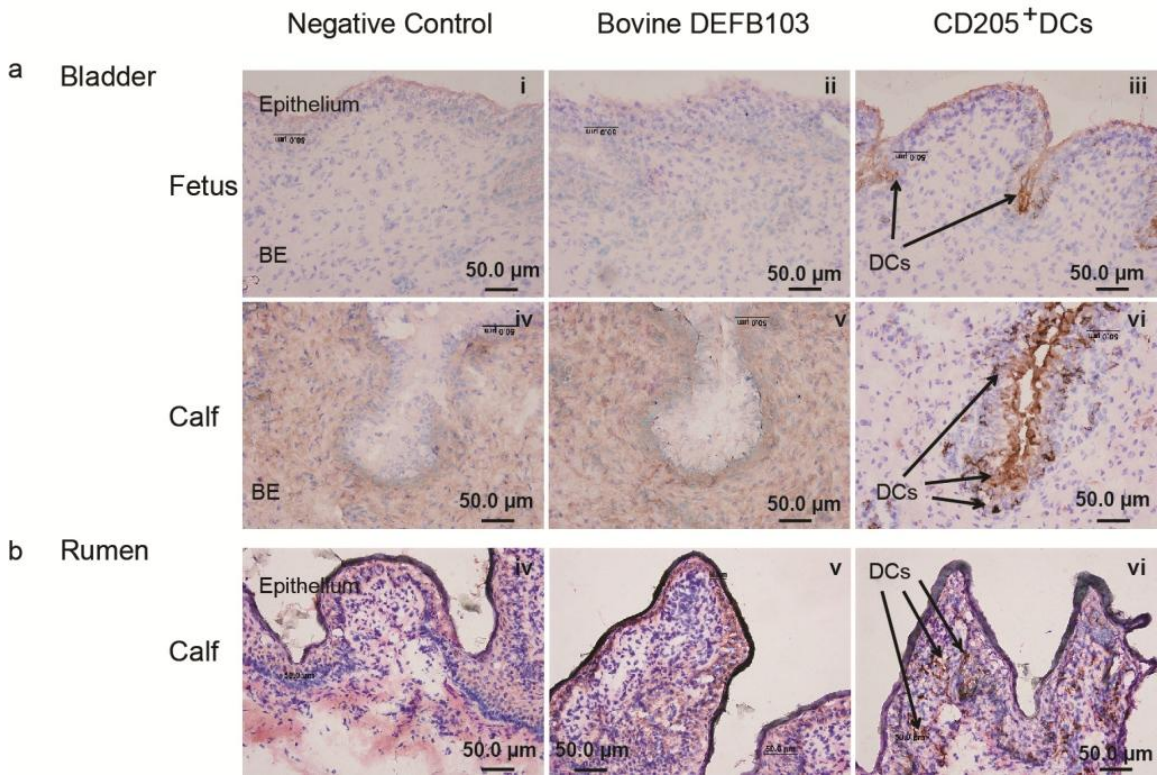


Figure 3.6: Immunohistochemical staining of **a** bladder and **b** rumen with irrelevant affinity-purified rabbit antisera specific to human CCR5 protein (Negative Control), affinity purified rabbit anti-bovine DEFB103, and a monoclonal antibody specific for CD205 protein expressed on dendritic cells (DCs). Staining presented is representative of that observed for tissues collected from three animals. *BE* basal epithelial cell layer at the junction between the epidermis and dermis; *Epi surface* outer keratinized surface of the epithelial tissue. Scale bars 50 µM

IHC staining of nictitating membrane and skin, two other stratified squamous epithelial tissues with abundant *DEFB103* transcript, revealed similar patterns of DEFB103 protein expression with some distinct tissue differences (Table 3.1). Nictitating membrane collected from three, 10- to 14-day-old calves consistently displayed visible staining for DEFB103 in basal epithelial cells but also throughout the entire epithelial layer, including stratified squamous epithelium (Figure 3.5b v; Table 3.1). In contrast, nictitating membrane collected from three fetuses displayed weak DEFB103 expression in the basal epithelial cell layer and throughout the entire epithelial layer (Figure 3.5b ii; Table 3.1). Selected areas of the panels in Figure 3.5 for both buccal epithelium and nictitating membrane collected from fetuses and 10- to 14-day-old calves were magnified to better reveal the marked differences in cellular staining patterns for DEFB103 protein in these tissues (Figure 3.7). Skin collected from both 10- to 14-day-old calves and fetuses displayed similar patterns of DEFB103 staining which were restricted primarily to the basal epithelial cell layer, including cells adjacent to the base and sides of hair follicles (Figure 3.5c ii and v; Table 3.1).

Rumen was the only gastrointestinal tissue with a level of DEFB103 transcript similar to other tissues containing stratified squamous epithelium (Figure 3.2a). Rumen epithelium consists of stratified squamous epithelium, rather than the simple columnar epithelium that is present in the small and large intestine. Rumen tissue collected from 10- to 14-day-old calves revealed specific but weak DEFB103 is staining throughout the epithelial layer (Figure 3.6b).

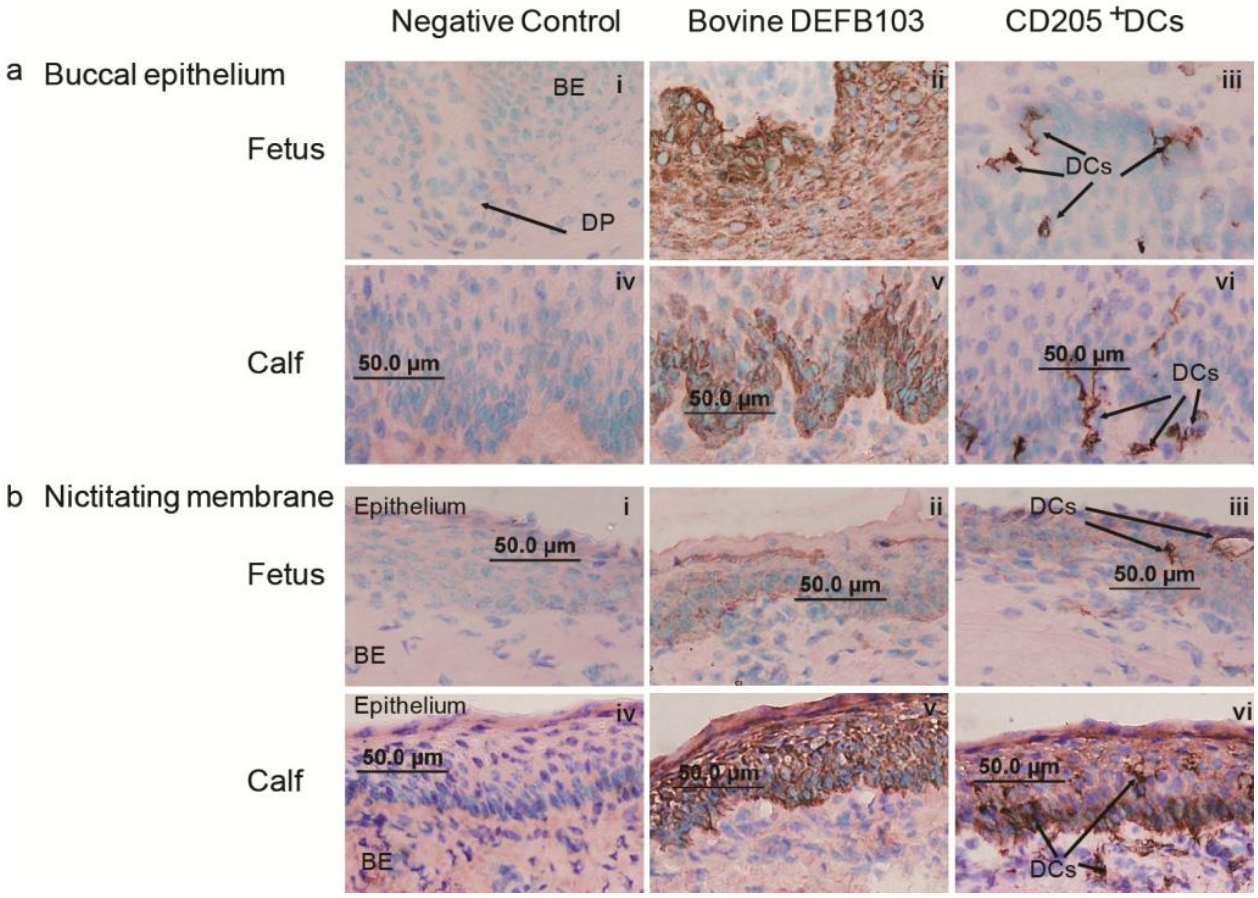


Figure 3.7: Magnified image of DEFB103 and CD205 staining in **a** buccal epithelium and **b** nictitating membrane. *BE* basal epithelial cell layer at the junction between the epidermis and dermis; *DP* dermal papillae; *Epi surface* outer surface of the epithelial cell surface. Arrows indicate DCs. Scale bars 50 µM

Table 3.1: IHC staining of DEEB103 protein and DCs in tissues with stratified squamous epithelium

Tissue	Cell layer	Age	DEFB103 Staining[b]	Dendritic cells[c]
Buccal epithelium[a]				
	Epidermis	Fetus	++; +; ++	+; +; +
		Calf	–; +; –	–; +; –
	Basal cells	Fetus	+++;+++;+++	+;+;+
		Calf	+++;+++;+++	++;++;+
	Dermis	Fetus	–;–;–	+;–;+
		Calf	–;–;–	+;+;+
Nictitating membrane[a]	Epidermis	Fetus	+;–;+	–;–;+
		Calf	+++;+++;+++	++;++;++
	Basal cells	Fetus	+;–;–	+;–;–
		Calf	++;+++;++	++;++;++
	Dermis	Fetus	–;–;–	–;–;+
		Calf	–;–;–	+;+;+
Skin[a]	Epidermis	Fetus	–; +; –	–;–;–
		Calf	+;+;+	–;–;–
	Basal cells	Fetus	++;++;+++	+;+;+
		Calf	+++;+++;+++	+;+;+
	Dermis	Fetus	–;–;–	+;+;+
		Calf	–;–;–	+;+;+

[a]Tissues were collected from 3 fetuses (7.5-8.5 months gestation) and three newborn calves (10-14 days old)

[b]Staining intensity for tissue sections from individual animals: – no visible staining; + faint visible staining; ++ distinct visible staining; +++ very strong staining

[c] Dendritic cell abundance in tissue sections from individual animals: – none visible; + rare positive cell; ++ numerous positive cells

IHC staining of $CD205^+$ DCs was completed to determine if there was a correlation between the pattern of DEFB103 expression in epithelial tissues and the localization or abundance of DCs (Figure 3.5a iii and vi). Numerous $CD205^+$ DCs were observed in close proximity to basal epithelial cells in buccal epithelium collected from both 10- to 14-day-old calves and fetuses (Figure 3.5a iii and vi; Figure 3.7a), where DEFB103 protein expression was abundant. In contrast, DC localization was very different in nictitating membranes collected from 10- to 14-day-old calves and fetuses (Figure 3.5b iii and vi; Figure 3.7 b; Table 3.1). Numerous DCs were associated with the basal epithelial cell layer and scattered throughout the entire epithelial layer of nictitating membranes collected from 10- to 14-day-old calves (Table 3.1). This pattern of DC distribution overlapped with the expression of DEFB103 protein throughout the epithelial layer of the nictitating membrane. In contrast, few $CD205^+$ DCs were present in fetal nictitating membranes where there was weak staining for DEFB103. A close association between basal epithelial cells and $CD205^+$ DCs was also observed in skin collected from both fetuses and 10- to 14-day-old calves (Figure 3.5c iii and vi). Furthermore, this association was present not only in areas of stratified squamous epithelium but also extended to the basal epithelium associated with hair follicles in the dermis, where DEFB103 protein was abundant in both fetuses and 10- to 14-day-old calves. Thus, the distribution and abundance of DEFB103 protein were consistent with the hypothesis that DEFB103 may play a role in DC recruitment in tissues with stratified squamous epithelium.

3.5 Discussion

Although little is known about the innate immune function of DEFB103, the expression of this gene has been analyzed in a wide variety of human and dog tissues. Harder et al. (2001) analysed the expression of the *HBD3* gene, the ortholog of *DEFB103*, in a wide variety of tissues

and reported 10 to 100-fold higher expression levels in skin and tonsils than all other tissues, including the tongue. Garcia et al. (2001) also used Real-Time qRT-PCR to analyzed *HBD3* expression in a wide variety of human tissues but did not analyze skin, tonsil, or tissues from the oral cavity. They reported the highest level of *HBD3* expression in placenta and testis, but it is not possible to directly compare their *HBD3* transcript abundance data with either Harder et al. (2001) or our current data. One marked contrast between our study and Garcia's results (2001), however, was the reported expression of *HBD3* in human neutrophils, whereas we found *DEFB103* expression was very low in bovine neutrophils (Figure 3.2b). Thus, significant interspecies differences may exist in tissue-specific *DEFB103* gene expression.

CBD103 gene expression has also been analyzed in dog tissues (Candille et al., 2007; Leonard et al., 2012). Leonard et al. (2012) studied *CBD103* gene expression in a limited number of tissues collected from dogs of an unspecified age and breed, and reported approximately 10^4 transcript copies per 10 ng total RNA in tongue and skin but very low mRNA transcript copy number (< 20 copies/10 ng RNA) in lung, bone marrow and epididymis. No transcript was detected throughout the gastrointestinal tract, kidney or pancreas. Similar to what was reported for dog tissues, we also observed that the *DEFB103* gene was highly expressed in a variety of oral cavity tissues such as buccal epithelium and tongue (Figure 3.2a) and other tissues with stratified squamous epithelium, such as nictitating membrane (third eyelid) and skin. Thus, it appears that in humans, dogs, and cattle the oral cavity and skin are important sites for *DEFB103* gene expression. Sorensen et al. (2003) reported that a combination of *transforming growth factor-α (TGF-α)* and insulin-like growth factor-I (IGF-I), both important in wound healing, can increase *HBD3* expression levels by 5-fold. DEFB103 may also play an important role in maintaining the epithelial barrier, especially at sites such as the oral cavity where abrasions can

occur while eating. This may be more pronounced in species, such as ruminants, that feed on forages and require repeated mastication to break down fibrous material.

Epithelial tissues from 10- to 14-day-old calves expressed similar levels of the *DEFB103* transcript as older animals, except buccal epithelium (Figure 3.3). Staining intensity for DEFB103 protein in buccal epithelium was very high in both 10- to 14-day-old and fetal calves but the pattern of DEFB103 staining changed markedly after birth (Figure 3.5a panels ii and v; Table 3.1). Thus, there may be a significant shift in the regulation of *DEFB103* expression in buccal epithelial cells between birth and two weeks of age. The first week of life postpartum has been characterised as a time of rapid changes in gene expression throughout the bovine gastrointestinal tract and many of these changes were correlated with the exposure to commensal microflora (Liang et al., 2014). This may be consistent with the hypothesis that DEFB103 plays a significant role, either through wound repair or DC recruitment, in maintaining the epithelial barrier. Similarly, infection, by an epitheliotropic viral also resulted in significantly ($P < 0.05$) increased *DEFB103* transcript abundance in the buccal epithelium (Figure 3.4b). Tissues were sampled at a single time point following viral infection, and further investigations will be necessary to determine when *DEFB103* expression is greatest. A collection of serial samples may determine if *DEFB103* expression increases early during infection when the virus is first causing epithelial cell destruction or after viral clearance when epithelial cell repair is occurring. These studies will provide greater insight into how external stimuli modulate *DEFB103* gene expression levels.

There are numerous reports that bacterial infections alter β-defensin gene expression in a variety of species and tissues. For example, bovine β-defensin 5 (DEFB5) gene expression increased in infected mammary glands (Goldammer et al., 2004) but no significant association

was found between *DEFB103* gene expression and nasal *Staphylococcus aureus* carriage status in humans (Fode et al., 2011). Ishimoto et al. (2006) reported that patients with pneumonia had higher serum HBD3 concentrations only during infection. Santoro et al. (2013) also reported that the level of *CBD103* transcript increases significantly in dogs with atopic skin versus healthy dogs. This finding, however, contradicts a previous study by Leonard et al. (2012). Increased *DEFB103* gene expression may, therefore, be a specific response to individual pathogens, sites of infection, or the age of an animal. Our current study did not implicate gender as a potential factor influencing *DEFB103* expression (Figure 3.4a), but the strength of this observation may be limited by the use of sexually immature animals and the comparison of females with castrated males.

IHC analysis of DEFB103 protein expression in a variety of tissues (Figure 3.5 ii and v) was consistent with the *DEFB103* gene expression data (Figure 3.2) and revealed that DEFB103 protein was expressed primarily in basal epithelial cells of stratified squamous epithelium but with some differences in distribution among epithelial tissues (Figure 3.5a and c; Table 3.1). Furthermore, production of the DEFB103 protein began during fetal development, prior to exposure to environmental antigens or infectious agents. The level of DEFB103 expression appeared to be much lower, however, in some fetal tissues, such as the nictitating membrane (Figure 3.5b ii; Table 3.1).

Leonard et al. (2012) also reported IHC staining of CBD103 in the canine skin but did not report localization of the protein to basal epithelial cells. Our current observation that DEFB103 protein was expressed primarily in basal epithelial cells may have significant implications for the recruitment and retention of DCs, such as Langerhans cells, in the skin. β-defensins have been implicated in DC recruitment (Diamond and Ryan, 2011) and specific DC subpopulations, such

as Langerhans cells, play a critical role in immune surveillance in the skin (Malissen et al., 2014). Bovine Langerhans cells establish a close relationship with epithelial cells in the skin of cattle (Bryan et al., 1988) but it is not known when or how DCs are first recruited to the skin.

Harvey et al. (2013) performed in vitro studies to confirm that human DEFB103 functioned as both a positive and negative regulator of cytokine and chemokine responses in both mouse and human myeloid DCs. The close association between $CD205^{+}$ DCs and basal epithelial cells in fetal and newborn calf stratified squamous epithelium (Figure 3.5 iii and vi; Figure 3.7; Table 3.1) suggests that DEFB103 may play a similar role in regulating DC recruitment and retention in the skin. Further studies will be required, however, to characterize the particular function that bovine DEFB103 may play in localisation of skin DCs and whether this activity is specific for individual DC subpopulations, such as Langerhans cells. Skin functions as potent immune induction site (Levin et al., 2014), and it will be important to determine which defensins play a crucial role in recruiting DCs to maintain immune surveillance and induce immune responses following infection.

4.0 IDENTIFICATION OF A NEW NON-CODING EXON AND HAPLOTYPE VARIABILITY IN THE CATTLE *DEFB103* GENE[3]

4.1 Abstract

The *DEFB103* gene is a member of the β-defensin gene family. In this study, we applied multiple sets of primers to characterize the *DEFB103* transcript. RT-PCR was used to determine the cDNA boundaries, and it indicated that the cDNA start point is at least 514 bp before the start codon and not further than 678 bp. Also, the length of the 3′UTR was determined to be at least 53 bp after the stop codon. Seven SNPs were located in the 5′UTR and comprised four different haplotypes in genomic DNA. This haplotype data could prove that at least two complete copies of *DEFB103* with an ATG start codon are present in cDNA in most cattle. In addition, haplotype data indicated that there are also multiple incomplete copies in most cattle. A non-coding exon 1a and a 261 bp intron 1a were identified in cattle and subsequently predicted in sheep and goats. *DEFB103* sequence assemblies and partial cloning sequences revealed two types of deletion (4-bp and 8-bp) in the 5′UTR. These observations could prove that these copies are not assembly artifacts.

4.2 Introduction

Defensins are members of a cationic protein family (3-4 kDa) with six conserved cysteine residues that create three cysteine disulfide bonds (Ganz and Lehrer, 1994). These conserved

[3] A version of this chapter has been published: Mirabzadeh-Ardakani, A., Griebel, P. and Schmutz, S.M. Identification of a new non-coding exon and haplotype variability in the cattle DEFB103 gene. *Gene* **551** (2014a), pp. 183-8.

cysteines provide three different patterns for the disulfide bonds, that divide the defensin family into three subfamilies called α, β (Lehrer and Ganz, 1999), and θ-defensins (Tang et al., 1999). β-defensins are in a single gene cluster in cattle, and they were mapped to chromosome 27 (Gallagher et al., 1995). β-defensin 103 (*DEFB103)* is a multi-functional gene influencing innate immunity (Bevins, 2006), coat color in dog (Candille et al., 2007), and body weight control in mice (Candille et al., 2007). All members of the β-defensin gene family in humans have two exons and one intron, with the exception of *DEFB105* (GenBank NM_152250.2) which has three coding exons and two introns (Pazgier et al., 2006).

Data from mRNA-seq showed that alternative splicing occurs in more than 90% of human genes (Pan et al., 2008; Wang et al., 2008). Genes with alternative promoter regions tend to generate alternative splicing (Xin et al., 2008). *Microphthalmia-associated transcription factor (MITF)* with nine exons (Tassabehji et al., 1994) and *Insulin-like growth factor 2 (IGF2)* with ten exons (Goodall and Schmutz, 2007) are such genes. In most genes with alternative splicing, some of the exons (Goodall and Schmutz, 2007) or part of the exons (Udono et al., 2000) are non-coding.

β-defensins are variable in their copy number (Hollox et al., 2003). *DEFB103* copy number variation has been reported in humans (2-12 copies per diploid genome) by Hollox et al. (2003) and in dogs (2-4 copies per diploid genome) by Leonard et al. (2012). Cattle *DEFB103* occurs in multiple copies in at least some individuals, based on sequence results from a cloning experiment (GenBank KM347983, KM347984, KM347985, and KM347986). Five SNPs were reported in the 5′UTR of the *DEFB103* by Dreger and Schmutz (2009) (c.-319A>G, c.-264C>T, c.-69A>G, c.-42A>G, and c.-34G>A), and two additional SNPs (c.-383A>G, c.-241G>A) were identified in the 5′UTR of the *DEFB103* (Mirabzadeh-Ardakani et al., 2014b). The current

research was conducted to investigate gene structure and haplotype variation of *DEFB103* in different tissues collected from dairy and beef cattle.

4.3 Materials and Methods

4.3.1 Sample collection

Animals used for this study were humanely killed according to Canadian Council on Animal Care guidelines and protocols approved by the University of Saskatchewan Animal Care Committee. Tissue samples were collected from seven neonatal Holstein calves and five 6- to 8-month-old Angus-cross beef calves. Tongue, buccal epithelium, conjunctiva, rumen, skin, trachea, and liver from newborn calves and tongue, buccal epithelium, trachea, and hard palate from Angus-cross calves were collected immediately after euthanasia and stored in RNA*later*® (Applied Biosystem-Ambion) in -80 ºC. Whole blood samples (5 ml) were collected from the jugular vein of each animal in a BD Vacutainers® with EDTA as an anticoagulant.

4.3.2 DNA and total RNA extraction

DNA was extracted from the buffy coat using an SQ blood DNA kit (OMEGA Bio-Tek, Inc.). Total RNA was extracted from collected tissues stabilized by RNA*later*® using the TRIzol method (Invitrogen). After the first step of extraction, the extracted RNA was treated with DNase-I (RNase-free; Invitrogen) to exclude any DNA contamination. The RNA quantity and quality were determined using a NanoDrop 2000 (NanoDrop, Technologies, DE, USA) spectrophotometer after the first extraction, and using an Agilent 2100 Bioanalyzer (G2938B, Agilent Technologies, Mississauga, Ontario) after the second RNA extraction. RNA with an integrity number higher than 7 was considered acceptable as intact RNA for cDNA preparation.

4.3.3 Reverse transcription and cDNA synthesis

Total RNA (250 ng) was reverse-transcribed using qScript™ cDNA SuperMix (Quanta Biosciences™), following the manufacturer's protocol. The cDNA was diluted to 1:10 as a template for the reverse transcription-polymerase chain reaction (RT-PCR).

4.3.4 Semi nested-PCR and RT-PCR amplification and genotyping

A stepwise walking procedure was used to design a 5′ forward primer approximately every 100bp. The furthest one that could amplify a fragment was selected for the experiment. Primers (Table 4.1) were designed using Primer3web (http://bioinfo.ut.ee/primer3/) except KNEST1-F and KNEST1-R primers (Dreger and Schmutz 2009). Since beta-defensin genes have multiple copies in cattle (Elsik et al., 2009), a semi nested-PCR was used for genomic DNA (gDNA) in an attempt to exclude various products or possible pseudogenes. In the primary PCR reaction, KNEST1-F forward and KNEST1-R reverse primers (Table 4.1) were used to amplify a 1925 bp fragment of genomic *DEFB103* that includes the 5′UTR, both previously reported exons, the intron, and the 3′UTR. The primary PCR product was used as a template for the semi-nested PCR. In the semi-nested amplification reaction, the same forward (KNEST1-F) and the KDEFEX1-R reverse primers (Table 4.1) were used to amplify the 5′UTR, and exon 1 (786 bp).

In the RT-PCR, three primer pairs (Table 4.1) were used to characterize the cDNA of the *DEFB103* gene. All the PCR products were separated using 1% agarose gels and then amplified products were extracted using a Gel-Extraction Kit (OMEGA Bio-Tek, Inc.). The extracted purified PCR products were sequenced (NRC-PBI, Saskatoon), and then analyzed using Sequencher software (version 4.8) to determine genotype and then haplotype for the seven SNPs in the 5′UTR of the *DEFB103* gene.

Table 4.1: Primers used in this study.

Primer Name	Starting bp[a]	Primer Sequence 5'-…-3'	Template	Product Size (bp)	Temp (°C)
KNEST1-F	-514	GCGTATCCATGCAGGTTCAG	Genomic	1925	63
KNEST1-R	*545	AGGCCTGAGGTCAGTCAGAACA			
KNEST1-F	-514	GCGTATCCATGCAGGTTCAG	Genomic	786	60
KDEFEX1-R	58+188	GATTAGTCTCTTAAGAGTGAATCAGAA			
KNEST1-F	-514	GCGTATCCATGCAGGTTCAG	cDNA	573 or 312	63
DEFEX1-R	38	CCTGGAACAGGCAGCAAGAACA			
DEFB103-5′UTR-3	-687	AGACACCTTCCTGACTCACG	cDNA	746	60
DEFEX1-R	38	CCTGGAACAGGCAGCAAGAACA			
KNEST1-F	-514	GCGTATCCATGCAGGTTCAG	cDNA	511	60
DEFB103Ex2-R2	*53	CTGGAGGAGGCATTTTCACG			

[a] Based on coding DNA reference sequence.

4.3.5 Haplotype determination

The five reported SNPs by Dreger and Schmutz (2009) formed two particular haplotypes. Two new SNPs in the 5′UTR increased the number of haplotypes from 2 to 4 (Mirabzadeh-Ardakani et al., 2014b). Two hundred and five Holstein cattle were genotyped for the seven SNPs in the 5′UTR of *DEFB103* (Mirabzadeh-Ardakani et al., 2014b). Haplotypes were initially identified in homozygous cattle. No animal presented a particular genotype that could not be explained by these four haplotypes (Table 4.2).

Table 4.2: Diploid haplotypes in genomic DNA.

Diploid haplotype	c.-383 A>G	c.-319 A>G	c.-264 C>T	c.-241 G>A	c.-69 A>G	c.-42 A>G	c.-34 G>A
1/1	AA	GG	TT	GG	GG	GG	AA
1/2	AA	GG	TT	AG	GG	GG	AA
1/3	AA	AG	CT	AG	AG	AG	AG
1/4	AG	AG	CT	AG	AG	AG	AG
2/2	AA	GG	TT	AA	GG	GG	AA
2/3	AA	AG	CT	AA	AG	AG	AG
2/4	AG	AG	CT	AA	AG	AG	AG
3/3	AA	AA	CC	AA	AA	AA	GG
3/4	AG	AA	CC	AA	AA	AA	GG
4/4	GG	AA	CC	AA	AA	AA	GG

4.4 Results

4.4.1 SNP haplotypes in genomic DNA

Cattle were first genotyped for the seven known SNPs in the 5′UTR of *DEFB103*. Four haploid haplotypes were determined for these seven SNPs (c.-383A>G, c.-319A>G, c.-264C>T, c.-241G>A, c.-69A>G, c.-42A>G, and c.-34G>A) in Holstein cattle (Mirabzadeh-Ardakani et al., 2014b) (Table 4.3). Although the four haploid haplotypes that were previously identified were present in both Holstein dairy cattle and beef cattle, in the calves studied in this experiment, only haploid haplotypes 2, 3, and 4 were present in the genomic DNA of the Holsteins, and only haploid haplotypes 2, and 4 were present in the genomic DNA of the beef calves.

4.4.2 Characterization of cDNA start and end positions

Two primer pairs (KNEST1-F and DEFB103Ex2-R2; DEFB103-5′UTR-3 and DEFEX1-R) (Table 4.1, Figure 4.1) were used to determine the start (in the 5′UTR) and the end (in the 3′UTR) of the *DEFB103* cDNA transcript in a tongue cDNA sample. A RT-PCR product (511 bp) was obtained using the KNEST1-F and the DEFB103Ex2-R2 primers. However, no fragment was obtained using the DEFB103-5′UTR-3 and the DEFEX1-R primers. The DEFEX1-R reverse primer had been designed in exon 1, and it must have a complementary site in the cDNA transcript. Therefore, the DEFB103-5′UTR-3 forward primer in the 5′UTR did not have a complementary site in the cDNA transcript. As a result, the start of the *DEFB103* transcript was determined to be at least 514 bp before the start codon (-514, Figure 4.1), and not further than 678 bp. The length of the 3′UTR was determined to be at least 53 bp after the stop codon (*53, Figure 4.1).

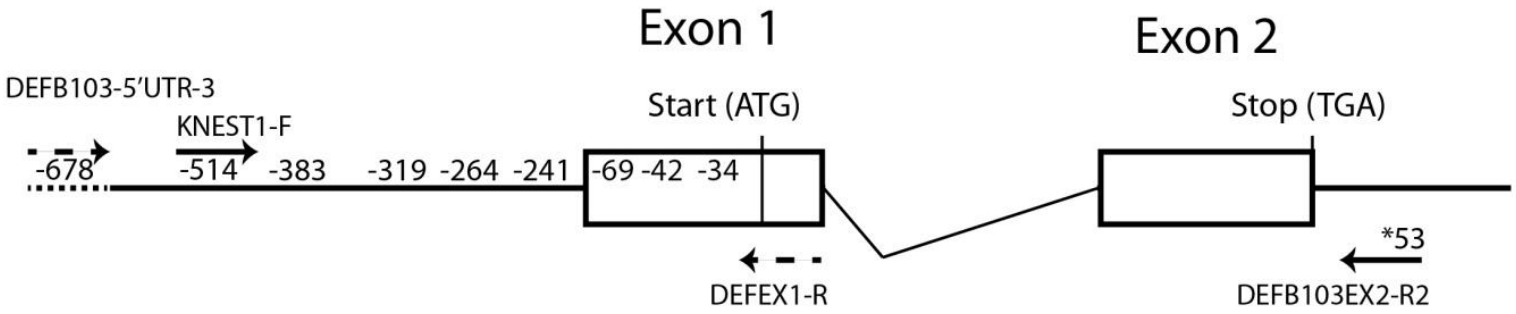


Figure 4.1: Characterization of the start and end points of the *DEFB103* cDNA transcript. Arrows with the same pattern indicate primer pairs used for this determination.

Table 4.3: Haploid haplotypes

		c.-383A>G	c.-319A>G	c.-264C>T	c.-241G>A	c.-69A>G	c.-42A>G	c.-34G>A
Haplotypes[a] (including intron 1A)	1	A	G	T	G	G	G	A
	2	A	G	T	A	G	G	A
	3	A	A	C	A	A	A	G
	4	G	A	C	A	A	A	G
		c.-383A>G	in intron 1a	in intron 1a	in intron 1a	c.-69A>G	c.-42A>G	c.-34G>A
Haplotypes[b] (intron 1a spliced out)	X	A	-	-	-	G	G	A
	Y	A	-	-	-	A	A	G
	Z	G	-	-	-	A	A	G

[a] Haplotypes derived from genomic DNA (from all cattle studied in this project) and partial copy cDNA (1-4)

[b] Haplotypes derived from partial and complete copies of cDNA (X-Z)

4.4.3 SNP haplotypes in cDNA

At the beginning of this study, KNEST1-F and DEFEX1-R primers (Table 4.1) were used to amplify a 573 bp fragment of *DEFB103* and genotype the seven SNPs in the 5′UTR. However, two PCR products (573 bp and 312 bp) were amplified by using cDNA from the oral cavity tissues (tongue, buccal, hard palate), conjunctiva, rumen, bladder, trachea and liver as a template (Figure 4.2a and b; Appendix B), but only one product was amplified (573 bp) from the skin and lung cDNA (Figure 4.2a). Both PCR products were sequenced and were aligned to genomic DNA. This allowed us to determine that a 261 bp fragment was spliced out in the 5′UTR of *DEFB103* in the 321 bp PCR product. At the 5′ end of intron 1a, there is a donor site (GT), and at the 3′ end of this fragment, there is an acceptor site (AG) for splicing (Figure 4.2b). Having these splicing sites before the first coding exon introduces a new intron (intron 1a) and a non-coding exon (exon 1a) before the first coding exon in cattle *DEFB103* (312 bp product) (Figure 4.2b). The same sequence was in the partial cDNA (573 bp), but splicing did not occur in this partial copy, which suggests that the partial copies of *DEFB103* were not all identical.

Sequences with intron 1a spliced out and including intron 1a had a different number of SNPs included because 3 of the SNPs (c.-319A>G, c.-264C>T, and c.-241G>A) were in the fragment that was intron 1a in some copies. Haplotypes in the partial cDNA product including intron 1a (573 bp) include all seven SNPs, therefore, their haplotypes were named like the genomic DNA haplotypes, by numbers (Table 4.3) whereas the haplotypes for copies that had intron 1a were named by letters (Table 4.3). The haploid haplotypes 1 and 2 in the genomic DNA are equivalent to haploid haplotype X, 3 is equivalent to Y, and 4 is equivalent to Z in partial cDNA product with the intron 1a (312 bp). Some cattle had more than one diploid

haplotype based on repeated PCR reactions, suggesting that a different copy was amplified on different occasions (Table 4.4).

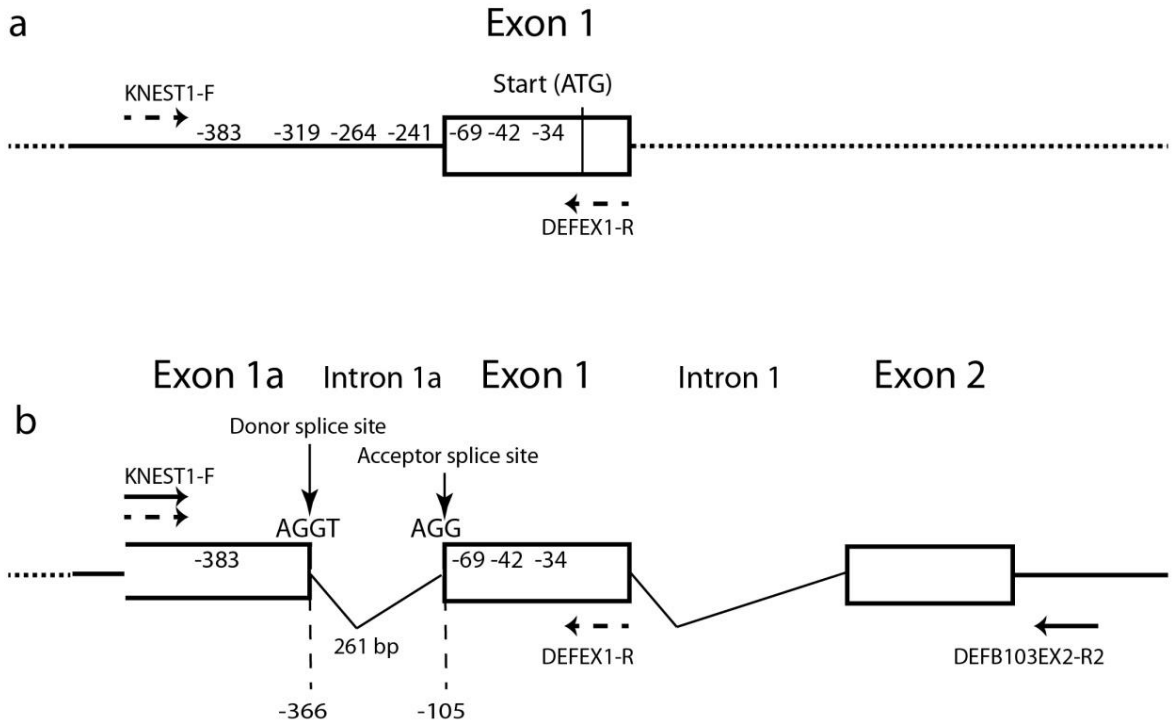


Figure 4.2: Observed forms of cDNA prepared from cattle RNA. a) A 573 bp partial cDNA (including intron 1a). b) A 312 bp partial cDNA when KNEST1-F and DEFEX1-R primers were used (intron 1a spliced out), and a 511 bp functional cDNA transcript when KNEST1-F and DEFB103EX2-R2 primers were used (intron 1a spliced out).

Table 4.4: Diploid haplotype in genomic DNA, partial cDNA (PCR by using KNEST1-F and DEFEX1-R primers), and the presumptive functional cDNA (PCR by using KNEST1-F, DEFB103EX2-R2 primers).

Haplotype from	Tissue	Holstein calves								Angus-cross calves				
		A	B	C	E	H	I	J	K	N	O	P	Q	T
gDNA	Blood	2/4	3/4	2/2	2/3	3/4	3/4	2/4	3/4	2/4	2/4	2/2	2/2	2/2
Partial cDNA (including intron 1a)	Bladder	1/4	1/4	1/4		1/4				-	-	-	-	-
	Liver	1/4	1/4	1/4		1/4	1/4			-	-	-	-	-
	Lung	1/4	1/4	1/4	1/2	1/4	1/4	1/2		-	-	-	-	-
	Rumen	1/4	1/4	1/4	1/2	1/4	1/4		1/2	-	-	-	-	-
	Rumen								3/4	-	-	-	-	-
	Skin	1/4	1/4	1/4	1/2	1/4	1/4	1/2		-	-	-	-	-
	Skin	3/4	3/4	3/4	3/4	3/4		3/4		-	-	-	-	-
	Conjunctiva						3/4			-	-	-	-	-
	Buccal	1/4	1/4	1/4	1/2	1/4	1/4	1/2		1/4	1/4	1/3	1/4	
	Tongue	1/4	1/4	1/4	1/2	1/4	1/4	1/2	3/4	1/4	1/4	1/4	1/4	2/4
	Tongue	2/4	3/4	3/4			3/4	3/4		3/4	3/4	2/4	3/4	
	Trachea	1/4	1/4	1/4	1/2	1/4	1/4	1/2		1/4	1/4	1/4	1/4	
	Trachea							3/4		3/4			3/4	
	Hard palate	-	-	-	-	-	-	-	-	1/4	1/4	1/4	1/4	2/4

Table 4.4: Continued

Haplotype from	Tissue	Holstein calves								Angus-cross calves				
		A	B	C	E	H	I	J	K	N	O	P	Q	T
Partial cDNA (intron 1a spliced out)	Bladder				X/Z					-	-	-	-	-
	Liver				X/Z			X/Z		-	-	-	-	-
	Liver				X/X					-	-	-	-	-
	Rumen	X/Z[a]	X/Z	X/X	X/X	X/Y	X/X			-	-	-	-	-
	Conjunctiva		Y/Z	X/X	X/X	X/Y	X/X			-	-	-	-	-
	Buccal	X/Z	Y/Z	X/X		Y/Z	Y/Z			X/Z	X/Z		X/X	X/X
	Tongue	X/Z	Y/Z	X/X	X/Z	X/Y	Y/Z	X/X	X/Y	X/Z	X/Z	X/X	X/X	X/X
	Tongue				X/X			X/Z						
	Trachea	X/Y		X/X	X/Z	X/X	X/Z					X/X		
	Hard palate	-	-	-	-	-	-	-	-			X/X		
Complete cDNA (intron 1a spliced out)	Rumen			X/X	X/X					-	-	-	-	-
	Skin	X/X		X/X	X/Y	Y/Z	X/Z	X/Z		-	-	-	-	-
	Conjunctiva		Y/Z	X/X	X/X	X/Z	Y/Z			-	-	-	-	-
	Buccal	X/Z	Y/Z	X/X	X/Y	Y/Z	Y/Z	X/X		X/Z	X/Z		X/X	X/X
	Tongue	X/X	Y/Z	X/X	X/X			X/Z	Y/Z	X/Z	X/Z	X/X	X/Z	X/X
	Tongue												X/X	
	Trachea	X/X		X/X	X/Y	X/X		X/X						
	Hard palate	-	-	-	-	-	-	-	-	X/Z	X/Z	X/X		X/X

[a] X/X in cDNA is equivalent with 1/1, 1/2, 2/2 in genomic DNA. X/Y is equivalent with 13, and 23. X/Z is equivalent with 14, and 24. Y/Y is equivalent with 33. Y/Z is equivalent with 34. Z/Z is equivalent with 44.

To study this further, the same forward (KNEST1-F) and DEFB103EX2-R2 reverse primers (Table 4.1, Figure 4.2b; Appendix C) were used to amplify a fragment that includes both previously known coding exons. Only one PCR product of 511 bp was amplified in all of the tissues, and this piece included intron 1a (Figure 4.2b). However, a second haplotype was often observed in individual animals when the PCR reaction was repeated. This observation suggests that more than one copy included three exons and two introns. Based on this evidence, we conclude the 511 bp cDNA represents a functional transcript of *DEFB103* (GenBank KJ461433) because of the presence of both coding exons in this transcript.

4.4.4 SNP haplotypes in genomic DNA versus cDNA

Haplotypes from the partial cDNA including intron 1a (573 bp) were not always consistent with the genomic haplotype based on DNA extracted from the blood of the same individual (Table 4.4). As an example, two haplotypes (1/4, and 3/4) were observed in skin and tongue in animal C, despite the fact that its haplotype in genomic DNA was 2/2. Haplotype results from partial cDNA with intron 1a spliced out (312 bp) for most of the tissues from a particular animal were consistent with their equivalent genomic haplotype (Table 4.4), with the exception of animal E. In the presumptive functional cDNA transcript that includes both coding exons and intron 1a, most of the tissues in all of the animals were consistent with their equivalent genomic haplotype. This suggests that more than one functional copy and multiple partial copies are present in *DEFB103,* and the CNV could explain this in defensins (Elsik et al., 2009).

4.4.5 DEFB103 genomic 5′UTR in different species

A 590 bp sequence from the 5′UTR of *Bos taurus* (cattle) *DEFB103* was used to BLAST the genomic DNA sequences from 8 different species: *Capra hircus* (goat), *Ovis aries* (sheep),

Equus caballus (horse), *Sus scrofa* (pig), *Canis lupus familiaris* (dog), *Tursiops truncatus* (bottlenose dolphin), *Mus musculus* (house mouse), and *Homo sapiens* (human). Among these species, sheep and goat were the only species that had a comparable sequence to the 5′UTR of cattle *DEFB103*. Three copies of *DEFB103* were observed in sheep and goats based on the BLAST output. The genomic homology of these copies varied from 86-94% for sheep and cattle and 85-93% for goat and cattle. Further analysis using Sequencher software (version 4.8) indicated that sheep and goat have the same splicing sites in their sequence, and, therefore, they should also have an intron 1a and non-coding exon 1a. The length of intron 1a is 260 bp in both sheep and goat, in comparison to the 261 bp in cattle.

4.5 Discussion

4.5.1 New DEFB103 gene structure

It is not uncommon for genes to have non-coding first exons. The tyrosinase-related protein 1 (*TYRP1*) gene is an example of a gene with a non-coding first exon (Box et al., 1998). A non-coding exon before the first coding exon of the *DEFB103* gene has not been previously reported in any species. There are two ATG codons in exon1a. The first ATG would introduce two stop codons (TGA, TAG) and the second one would introduce one stop codon (TAG) before exon 1. This is consistent with our interpretation that exon1a is a non-coding exon. The poly A tail was not detected in this study, and, therefore, the 3′UTR is longer than 53 bp. The length of the 3′UTR varied from 60 nucleotides to about 4000 nucleotides in mammals, and on average it is longer than the 5′UTR (Hesketh, 2001). A new gene structure is proposed for the *DEFB103* gene in cattle (Figure 4.3), based on the current study. We also predict that sheep and goats would have three exons. Common evolution in these ruminants may explain the similarity in the *DEFB103* 5′UTR sequences.

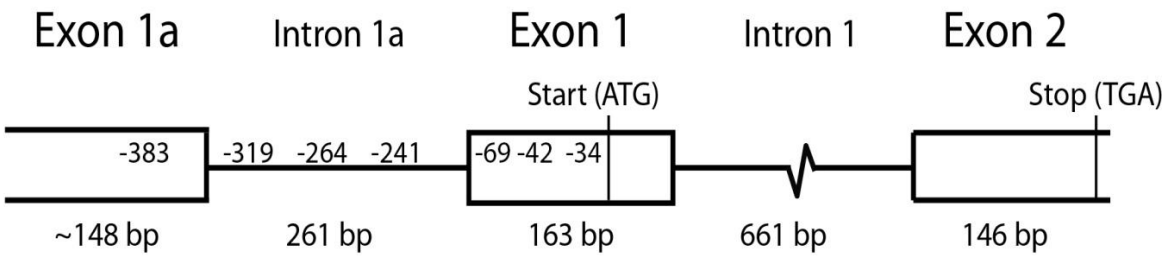


Figure 4.3: Proposed cattle *DEFB103* gene structure with a non-coding exon (exon 1a).

4.5.2 Alternate splicing site

Alternate splicing is common in more than 90% of human genes (Pan et al., 2008; Wang et al., 2008). The 573 bp partial cDNA had the same splicing site that the 312 bp partial cDNA had, but intron 1a was not spliced out in the 573 bp partial cDNA.

The *microphthalmia-associated transcription factor (MITF)* gene is involved in multiple pathways, such as pigmentation and deafness disorders in humans (Smith et al., 2000; Steingrimsson et al., 2004) and coat color or pattern in mice (Moore, 1995; Widlund and Fisher, 2003), cattle (Philipp et al., 2011) and dogs (Schmutz et al., 2009). *MITF* has nine exons (Tassabehji et al., 1994), but multiple promoters and splicing of alternative first exons generate multiple isoforms of *MITF* (Amae et al., 1998; Yasumoto et al., 1998). Among these isoforms, *MITF-M* has two alternate acceptor sites at the 5′end of exon 6 (3′ end of intron 5) in humans (Amae et al., 1998; Yasumoto et al., 1998), mice (Steingrimsson et al., 1994), dogs (Schmutz et al., 2009) and cattle (Strom, 2003). These are called *MITF-M+* and *MITF-M−* (Goding, 2000).

Alternative splicing was also reported in cattle *Insulin-like growth factor 2*. *IGF2* has ten exons, and among these, the first seven exons are non-coding (Goodall and Schmutz, 2007). Seven isoforms were reported for the *IGF2* transcript indicating multiple promoters involved in

IGF2 transcription. One of the transcripts (exon 1-3, 8-10), had an alternative donor splicing site 41 bp downstream from exon two, that is exclusive to liver tissue (Goodall and Schmutz, 2007).

Both amplicons of *DEFB103* (573bp and 312bp) like *MITF-M* (*MITF-M*+ and *MITF-M*−) and *IGF2* (*IGF2*+41bp and *IGF2*) represent the alternate acceptor (Strom, 2003) and donor (Goodall and Schmutz, 2007) sites, and they amplified simultaneously by PCR.

4.5.3 DEFB103 copies in two published assemblies versus RT-PCR results

Two assemblies are available for *Bos taurus* genomic sequence in GenBank (Btau 4.6.1, and UMD 3.1). *DEFB103* sequence previously obtained by Dreger and Schmutz (2009) (GenBank EU715240.1) was used to BLAST against both *Bos taurus* genome sequences (NCBI – Annotation Release 103). The BLAST results from these two annotations were analyzed, and each copy of this gene was localized based on its predicted position (Figure 4.4).

The Btau 4.6.1 assembly had two complete copies with an ATG start codon, and the rest of the complete copies had ATA instead of an ATG (Figure 4.4). In the Btau 4.6.1 assembly, all of the eight recognized copies included exons 1 and 2 and intron 1 while the UMD 3.1 assembly had six complete copies, only one of which had an ATG start codon, and multiple incomplete copies. The sequence of the third incomplete copy in the UMD 3.1 assembly does not match with any sequence obtained in this study. Therefore, it is likely a sequencing artifact. Three different small deletions were identified in one or more different copies that began with ATA of these assemblies. Among these deletions c.-454_-457delACAC in UMD copy 10 and BTAU copies 6 and 8; and c.-199_-206delTAATTAAG in UMD copy 11 and BTAU copies 5 and 7, were present in the partial copies obtained by previous cloning experiments (GenBank KM347983 and KM347984). Therefore, they are probably not sequencing artifacts. These deletions do not occur simultaneously. In addition, c.-27_-40delACACCGTCCTGTCC occurred in UMD copy 12.

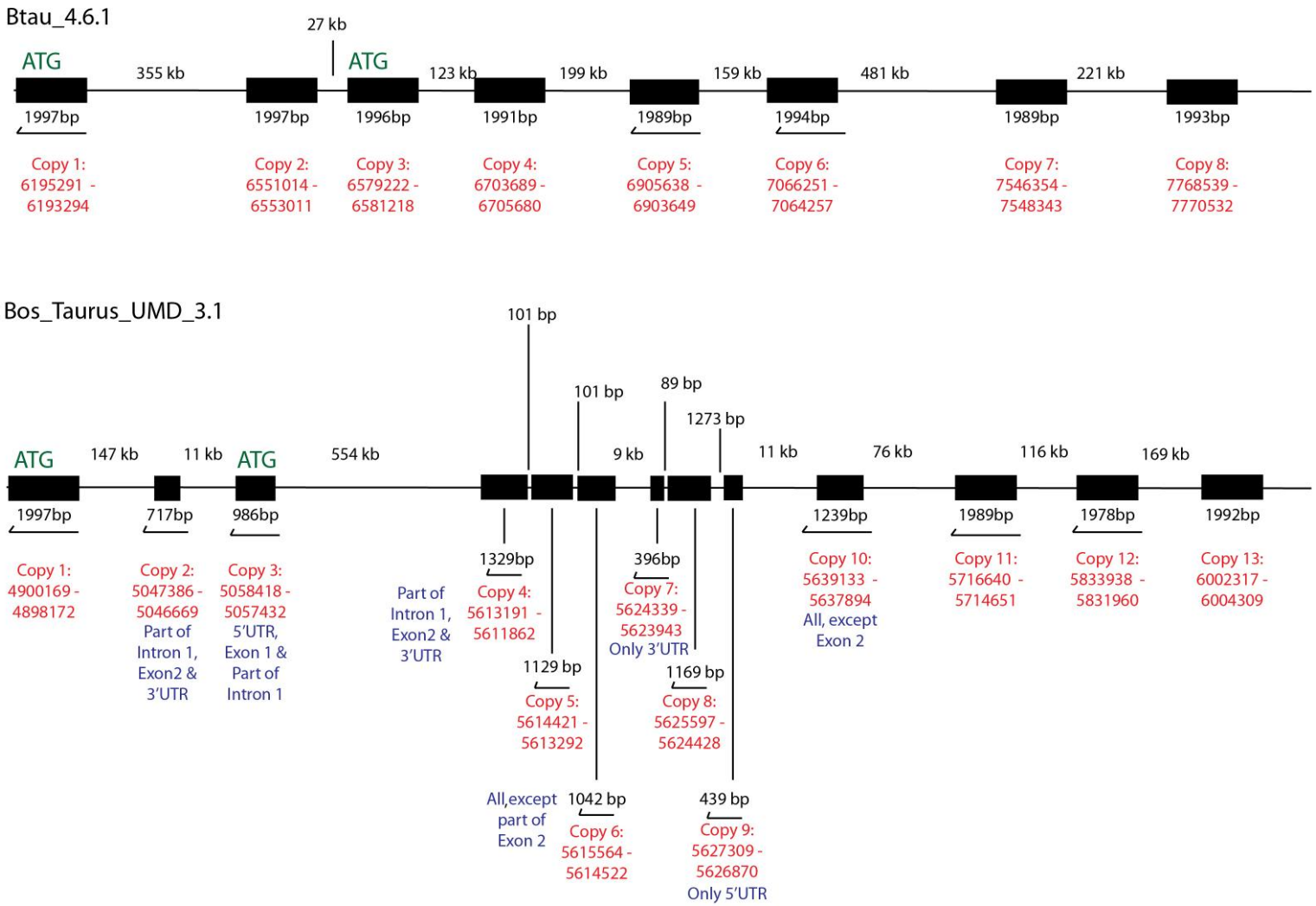


Figure 4.4: Schematic presentations of the location of different *DEFB103* copies in the two assemblies (Btau_4.6.1, and Bos_Taurus_UMD_3.1; Annotation Release 103). Arrows indicate the orientation of the sequences.

In the PCR in this study, using KNEST1-F (forward) and DEFEX1-R (reverse) primers, the reverse primer was located at the end of exon 1, and two PCR products with an ATG start codon (Figure 4.2a-b) were amplified using these primers. These products might be from a complete or partial copy of the gene. These data support both the Btau 4.6.1 and the UMD 3.1 assemblies because both of them have two copies with ATG. However, in the PCR using the KNEST1-F (forward) and the DEFB103EX2-R2 (reverse) primers, only one PCR product (511 bp) was amplified with an ATG (Figure 4.2b). In this case, the only assembly that could be supported is the UMD 3.1 because it has only one complete copy with an ATG start codon. Some of the haplotypes from the partial and functional cDNA transcripts are inconsistent with the genomic haplotype from the same individual. Since the *DEFB103* gene occurs in multiple copies in cattle (Dreger and Schmutz, 2009; Elsik et al., 2009), amplifying a different copy or a pseudogene is probably the reason for the inconsistency. Since the complete cDNA transcript from most of the tissues had the equivalent genomic haplotype in most cattle, we conclude that the fragment amplified using genomic DNA was from one of the complete copies, which could be functional copies.

Five cattle (i.e. A, E, I, J, and Q) had two functional copies with different haplotypes, and animal H had three. Based on these observations, at least, two complete transcripts of *DEFB103* are present in the cattle genome (Table 4.4). Six cattle (i.e. B, C, N, O, P, and T) had only one haplotype for different tissues. Cattle with one haplotype may still have two or more complete or functional copies, but they are just homozygous for that haplotype. The presence of two copies supports the Btau 4.6.1 assembly, because it has two complete copies, with an ATG start codon. However, our results are consistent with parts of both assemblies for the *DEFB103* region. So in reality, the assembly for *DEFB103* might be a mixture of both published assemblies. High

Fidelity Duplication (HFDs) regions, >5kb with >99% sequence homology, were identified in both assemblies (Zimin et al., 2012), with 3111 HFDs in Btau 4.2 in comparison to 69 in UMD 3.1. So the eight complete copies could be one of the HFDs in the Btau 4.6.1 assembly.

In conclusion, two or multiple haplotypes in different tissues in one individual prove that *DEFB103* has, at least, two partial copies and two complete copies with an ATG start codon. All of the complete copies have a non-coding exon (exon 1a), but not all of the partial copies.

5. DEFB103 GENOTYPE AND HAPLOTYPE ASSOCIATION STUDIES

5.1 Introduction

The *β-Defensin* gene family encodes small cationic peptides with antimicrobial activity (Ganz, 2003a) that play an important role in the innate immune system (Hazlett and Wu, 2011). *DEFB103* is a member of the *β-Defensin* genes cluster (Hardwick et al., 2011).

Genetic variation in immunopeptides could potentially lead to differences in disease susceptibility and/or resistance. In dairy cattle, combined defensin band patterns were determined by using PCR-RFLP of different *β-Defensin* genes. An association with low somatic cell count was reported (Ryniewicz et al., 2003). Since somatic cell count is an indicator of udder health (Mrode et al., 1998), members of the *β-Defensin* gene family could be genetic markers of udder susceptibility to inflammation, commonly known as mastitis. Mastitis is a serious and ongoing problem in dairy cattle (Thompson-Crispi et al., 2014a). Although many forms of mastitis respond well to antibiotic treatment, mastitis caused by *Staphylococcus aureus* does not (Royster and Wagner, 2015). Two populations of Holstein cattle that had been exposed to *S. aureus* provided an opportunity to study disease resistance and susceptibility in these animals.

Shipping fever continues to be a serious health hazard in beef cattle (Apley, 2014; Ng et al., 2015). It was reported as a reason for 70-80% of illness and 40-50% of death in USA's feedlots (Hilton, 2014). Shipping fever is commonly caused by bacteria such as *Mannheimia haemolytica*, or viruses such as bovine herpesvirus-1 (BHV-1) (Hodgson et al., 2012; Griebel et al., 2014). A challenge trial using these two agents was conducted (Griebel et al., 2014) and we

had the opportunity to collect samples from 60 crossbred calves to study potential resistance and/or susceptibility relative to their *DEFB103* haplotypes.

Ocular Squamous cell carcinoma or "Cancer-eye" commonly occurs on the epithelial surfaces of the conjunctiva (Tsujita and Plummer, 2010). Cancer-eye is more prevalent in cattle breeds with the unpigmented skin around the eyes (Stewart et al., 2006), such as Hereford. It typically has a late onset, with most affected cattle being greater than 7-8 years of age (Tsujita and Plummer, 2010). It is a rapidly developing cancer that often results in the necessity of humane euthanasia within months. Our tissue survey of *DEFB103* gene expression revealed epithelial tissues, including the conjunctiva, have the highest level of *DEFB103* gene expression (Chapter 3), and it was a motivation to study these diseases further.

Five SNPs (c.-319A>G, c.-264C>T, c.-69A>G, c.-42A>G, and c.-34G>A) had been reported in the 5′UTR of *DEFB103* (Dreger and Schmutz, 2009) and two other SNPs were discovered in the present study. The purpose of the current study was to evaluate the association of these *DEFB103* SNPs and haplotypes on *Staphylococcus aureus* mastitis in two herds of Holstein cattle, Cancer-eye in Hereford cattle, and shipping fever in Angus-crossed cattle.

5.2 Materials and Methods

5.2.1 Cattle Populations

5.2.1.1 Mastitis Herds

The Saskatchewan population (n=113) was composed of Holstein cattle from three herds. *S. aureus* prevalence for these herds was 3.9%, 14.5%, and 42.3% (Robbins, 1992). The Pennsylvania population, consisting of four herds (n=92; extracted in 1992), was used as the validation population. In previous related studies, these herds were chosen for their high

incidence of mastitis, and their management practices that exposed all cows to the risk of infection. Milk samples (3-5 ml) had been collected from each quarter of each cow, twice, with a one-month interval. Cows that were infected with *S. aureus* on both samplings were classified as having mastitis.

5.2.1.2 Hereford Cattle (Cancer-eye)

We had 49 stored DNA samples collected from cattle diagnosed with Cancer-eye at slaughter but did not have appropriate age-matched controls. Therefore, our study was focused on determining if a particular *DEFB103* genotype or haplotype predominated in this group. We also used DNA samples from 40 beef cattle (10 animals each from four major beef cattle breeds: Angus, Hereford, Simmental, Charolais) without Cancer-eye with different ages. Cancer-eye was identified by an inspecting veterinarian at a slaughter facility.

5.2.1.3 Viral and Bacterial Challenged Calves (shipping fever)

Sixty Angus-cross cattle, 6-8 months of age, were challenged with *Mannheimia haemolytica*, and bovine herpesvirus-1 (BHV-1) (Hodgson et al., 2012; Griebel et al., 2014). Nasal secretions were analyzed for interferon-gamma (IFN-γ) levels by capture ELISA (Raggo et al., 2000; Griebel et al., 2014). The level of serum haptoglobin also was quantified by capture ELISA to verify the response to BHV-1 infection (Godson et al., 1995; Griebel et al., 2014). Animals that survived the challenge were euthanized on day 14. DNA was extracted from 59 blood samples by using an SQ blood DNA kit (OMEGA Bio-Tek, Inc.).

5.2.2 Primer Design and PCR

β-Defensin genes have multiple copies in cattle (Elsik et al., 2009). A semi nested-PCR was used in an attempt to exclude various products or possible pseudogenes. In the primary PCR reaction, KNEST1-F forward and KNEST1-R reverse primers (Table 5.1) (Dreger and Schmutz, 2009) were used to amplify a 1923 bp fragment of *DEFB103* that includes the 5′UTR, both previously reported exons, the intron, and the 3′UTR. The primary PCR product was extracted from a 1% agarose gel (OMEGA Bio-Tek, Inc.). Then the extracted purified primary PCR product was used as a template for the semi-nested PCR. In the semi-nested amplification reaction, the same forward (KNEST1-F) and the KDEFEX1-R reverse primers (Table 5.1) were used to amplify the 5′UTR, and exon 1 (786 bp). The Kdefex1-R was designed using Primer3web (http://bioinfo.ut.ee/primer3/).

These fragments of *DEFB103* were amplified using PCR in 15µl reactions. The PCR reaction contains 1.5 µl of 10x PCR Buffer (Fermentas, Thermo Fisher Scientific Inc.), 0.3 µl of 10 mM dNTP, 0.9 µl of 25 mM $MgCl_2$, 10 pmol/µl each of forward and reverse primers (Operon), 0.1 µl of 5U/µl *Taq* polymerase (Fermentas, Thermo Fisher Scientific Inc.), 9.2 µl dH_2O, and 1 µl of ~50 ng/µl DNA template. PCR reactions were incubated at 95 ºC for 5 min (initial denaturation), and then followed by amplification for 35 cycles of 95 ºC for 45 s, different annealing temperature for each set of primers (Table 5.1) for 45 s, extension at 72 ºC for 1 min, and final extension at 72 ºC for 5 min using Stratagene Robocycler Gradient40 machines. All the PCR products were separated using 1% agarose gel and then amplified products were extracted using gel extraction kit (OMEGA Bio-Tek, Inc.). The obtained purified PCR products were sequenced at the National Research Council of Canada Plant Biotechnology

Institute (NRC-PBI, Saskatoon) using an ABI 3730xl DNA Analyzer (Applied Biosystems®), and The BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®).

Table 5.1: Primers used for genomic DNA.

Primer Name	**Starting bp**[a]	**Primer Sequence 5'-…-3'**	**Product Size (bp)**	**Annealing Temperature (°C)**
KNEST1-F	-514	GCGTATCCATGCAGGTTCAG	1925	63
KNEST1-R	*545	AGGCCTGAGGTCAGTCAGAACA		
KNEST1-F	-514	GCGTATCCATGCAGGTTCAG	786	60
KDEFEX1-R	58+188	GATTAGTCTCTTAAGAGTGAATCAGAA		

[a] Based on coding DNA reference sequence. *indicates the starting point is after the stop codon

5.2.3 Genotyping and Haplotype Identification

Five SNPs (c.-34G>A, c.-42A>G, c.-69A>G, c.-264C>T, and c.-319A>G) were previously reported at the 5′ UTR of cattle *DEFB103* (Dreger and Schmutz, 2009). Semi-nested PCR product sequences were aligned using the Sequencher software (version 4.8, Gene Codes Corporation, Ann Arbor, MI USA) to genotype cattle for each SNP. Haploid haplotypes were determined based on the genotype of the SNPs at the 5′ UTR.

5.2.4 Statistical Analysis

5.2.4.1 Mastitis Herds, and Hereford Cattle (Cancer-eye)

GraphPad Prism, version 6.01 was used to analyze the data (GraphPad Software, Inc., San Diego, CA). A two-tailed Fisher Exact test was used to determine if there was a significant difference in the presence or absence of *S. aureus* among the cattle of each diploid haplotype. To determine if a particular haplotype might be acting as a dominant allele to impart resistance or susceptibility to *S. aureus*, a Fisher Exact test was performed to compare the number of individuals with each haplotype versus those individuals who did not have that haplotype, with or without mastitis. Relative risk (RR) and odds ratios (OR) (95% confidence interval) were calculated. Relative risk and/or odds ratios are often reported for a particular genotype or haplotype that may impart some level of resistance to a disease (Lang and Secic, 2006). The Fisher exact test also was used to verify whether a particular *DEFB103* haplotype is associated with the relative risk of Cancer-eye. $P<0.05$ was considered statistically significant.

5.2.4.2 Viral and Bacterial Challenged Calves (shipping fever)

GraphPad Prism, version 6.01 was used to analyze the data (GraphPad Software, Inc., San Diego, CA). A Chi-Square test was used to verify whether there is a significant difference between survives and death rate in different haplotypes. A one-way ANOVA was used to verify whether there is a significant difference between the level of serum haptoglobin (μg/ml), and interferon-gamma (IFN-γ) (ng/ml) in steers (n=33) and heifers (n=26) with different diploid haplotype. An unpaired t-test was used to test whether there is a significant difference between the level of serum haptoglobin (μg/ml) and interferon-gamma (IFN-γ) (ng/ml) in the different diploid haplotype.

5.3 Result

5.3.1 Mastitis Herds

Two novel SNPs (c.-241G>A, and c.-383A>G) were identified in this study (Genbank KF545952.1). Four haplotypes were determined based on the five SNPs that had been reported by Dreger and Schmutz (2009) and these two new SNPs (Table 5.2).

There was a trend toward increased risk of mastitis in cows with the 1/4 diploid haplotype (P = 0.053), in the Saskatchewan population, when compared to individuals with other haplotypes (Table 5.3). In the Pennsylvania population, there was a trend toward decreased risk of mastitis in cows with the 4/4 diploid haplotype (P = 0.06), when compared to other haplotypes (Table 5.3). Therefore the results of these two studies are not consistent and therefore can not be considered as a significant finding in either case.

Table 5.2: Haploid haplotypes in genomic DNA and cDNA.

		c.-383 A>G	**c.-319 A>G**	**c.-264 C>T**	**c.-241 G>A**	**c.-69 A>G**	**c.-42 A>G**	**c.-34 G>A**
Genomic and Partial cDNA haplotypes (including intron 1a)	1	A	G	T	G	G	G	A
	2	A	G	T	A	G	G	A
	3	A	A	C	A	A	A	G
	4	G	A	C	A	A	A	G

In the Saskatchewan population, the number of individuals that had, at least, one copy of haplotype 2 (P = 0.09) showed a trend toward decreased risk of mastitis. On the other hand, the number of individuals that had, at least, one copy of haplotype 4 (P = 0.09), showed a trend toward increased risk of mastitis (Table 5.4). The relative risk and the odds ratios results (Table

5.4) indicate that individuals with *S. aureus* infection in the Saskatchewan population, with haploid haplotypes 2 and 3, and in the Pennsylvania population with haploid haplotypes 1 and 4 were less susceptible to mastitis. There was no consistent diploid or haploid haplotype that imparted resistance or susceptibility to *S. aureus* between the two populations.

Table 5.3: The *DEFB103* diploid haplotypes in cattle with *S. aureus* mastitis. Two-tailed P-values are reported based on a Fisher Exact test.

Haplotype	Saskatchewan Population			Pennsylvania Population		
	Mastitis	**No Mastitis**	**P**	**Mastitis**	**No Mastitis**	**P**
1/1	1	1	0.49	0	0	-
1/2	3	8	0.99	1	1	0.37
1/3	1	4	0.99	0	2	0.99
1/4	4	2	0.05	0	3	0.99
2/2	2	12	0.34	4	7	0.23
2/3	0	7	0.19	3	8	0.69
2/4	7	19	0.99	6	24	0.99
3/3	1	3	0.99	0	3	0.99
3/4	7	14	0.60	5	12	0.33
4/4	6	11	0.56	0	13	0.06
Total	32	81		19	73	

Table 5.4: The *DEFB103* haploid haplotypes of cattle with and without *S. aureus* mastitis.

Haplotype	P-value[a]	Mastitis		No Mastitis		Relative Risk (95% CI)	Odds Ratio (95% CI)
		Yes	No	Yes	No		
Saskatchewan Population							
1	0.31	9	23	15	66	1.52 (0.74-3.11)	1.72 (0.66-4.47)
2	0.09	12	20	46	35	0.66 (0.41-1.07)	0.46 (0.20-1.06)
3	0.66	9	23	28	53	0.81 (0.43-1.53)	0.74 (0.30-1.82)
4	0.09	24	8	46	35	1.32 (1.00-1.74)	2.28 (0.92-5.69)
Pennsylvania Population							
1	0.99	1	18	6	67	0.64 (0.08-5.00)	0.62 (0.07-5.49)
2	0.19	14	5	40	33	1.34 (0.96-1.89)	2.31 (0.75-7.08)
3	0.59	8	11	25	48	1.30 (0.66-2.28)	1.40 (0.50-3.92)
4	0.28	11	8	52	21	0.63 (0.54-1.22)	0.56 (0.20-1.57)

[a]Two-tailed P-values are reported based on the Fisher Exact test.

5.3.2 Cancer-eye Cattle

The six single nucleotide polymorphisms (SNPs) (c.-34G>A, c.-42A>G, c.-69A>G, c.-264C>T, c.-319A>G, and -383A>G) were identified in the 5'UTR of *DEFB103* in beef cattle. The c.-241G>A SNP was not polymorphic in this group of cattle. These six SNPs occurred in two haplotypes (2 and 4, Table 5.2) in the 49 Cancer-eye DNA samples from Hereford cattle. Haplotype and allelic frequencies were calculated for cattle affected with Cancer-eye and healthy beef cattle. All possible combinations of *DEFB103* haplotypes were identified among the samples (2/2, 2/4, and 4/4, Table 5.5). The Fisher exact test was used, and no significant difference (P = 0.43) in haplotype frequencies was observed among the 49 Hereford cattle with cancer-eye when compared to 40 healthy beef cattle (10 animals each from four major beef cattle breeds: Angus, Hereford, Simmental, Charolais). The minor allele frequency for SNPs in Cancer-eye samples was 26%, and in healthy beef cattle was 33.75%. Although the 40 "control" samples were from cattle of various ages, we conclude that sampling age-matched healthy Hereford cattle samples as a control group would not alter the *DEFB103* allele frequency in animals free of Cancer-eye.

Table 5.5: Haplotype frequencies in Hereford cattle with Cancer-eye and healthy beef cattle (10 animals from four major beef cattle breeds: Angus, Hereford, Simmental, Charolais).

Diploid	**Hereford cattle with Cancer-eye**		**Healthy beef cattle**	
Haplotype	**n**	**Frequency**	**n**	**Frequency**
2/2	27	0.55	19	0.47
2/4	19	0.39	15	0.38
4/4	3	0.06	6	0.15
Total	49		40	

5.3.3 Viral and Bacterial Challenged Calves (shipping fever)

The seven SNPs (c.-34G>A, c.-42A>G, c.-69A>G, c.-241G>A, c.-264C>T, c.-319A>G, and -383A>G) were identified in the 5'UTR of *DEFB103* in Angus-crossed cattle. These seven SNPs occurred in three haplotypes (1, 2 and 4, Table 5.2) in the 59 Angus-crossed cattle. All possible combinations of *DEFB103* haplotypes were identified among the samples (Table 5.6) except 1/4 diploid haplotype. Very few cattle were identified with the 1/1, 1/2, and 4/4 diploid haplotypes (Table 5.6). Because of their low frequency, only cattle with 2/2 and 2/4 diploid haplotypes were used in the analyzes.

Table 5.6: Diploid Haplotype frequency in the Angus' calves.

Diploid Haplotype	Number	Frequency
1/1	1	0.02
1/2	3	0.05
2/2	23	0.39
2/4	30	0.51
4/4	2	0.03

5.3.3.1 Survival Post Infection

A Chi-Square test indicated that there was no significant difference in survival time between steers (n=33) and heifers (n=26) with a 2/2 or 2/4 diploid haplotype (P=0.93). Steers and heifers were pooled for further analysis. A Log-rank (Mantel-Cox) indicated that there was no significant difference in the survival curve between cattle with diploid haplotypes 2/2 and 2/4 (P=0.50; Figure 5.1).

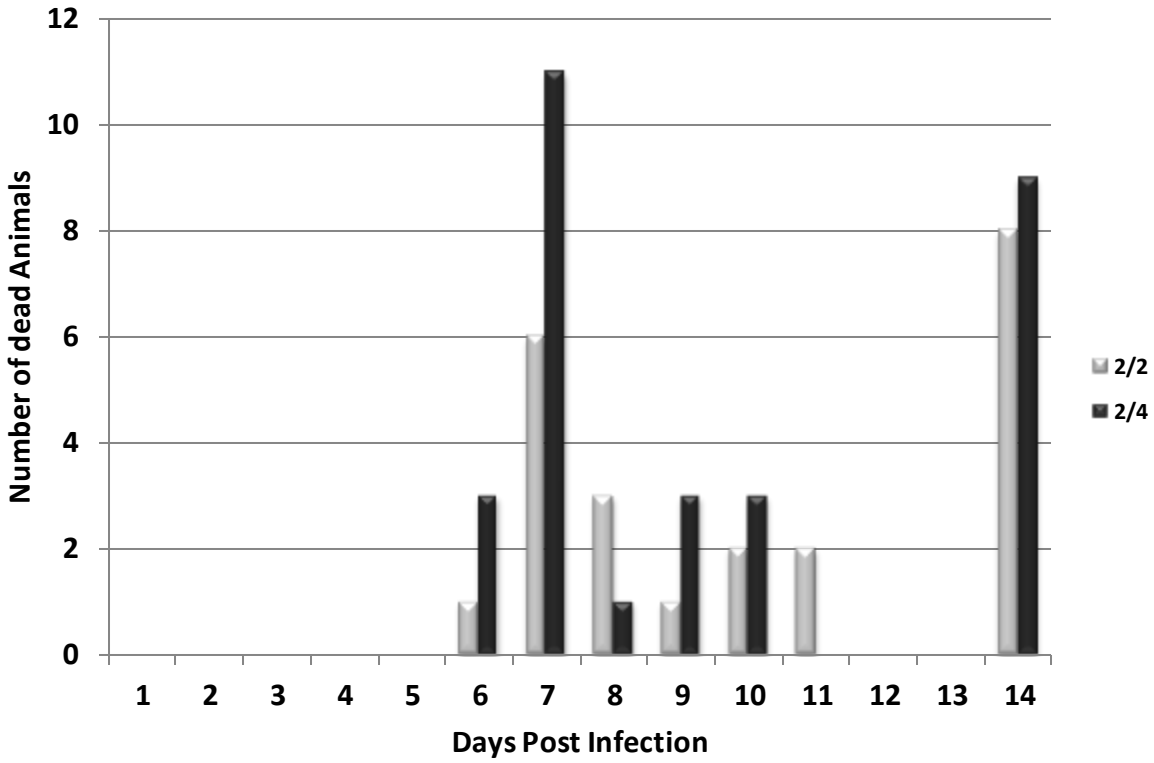


Figure 5.1: Survival Post-Infection. Cattle on the day 14th survived.

5.3.3.2 Levels of serum haptoglobin (µg/ml) and interferon-gamma (IFN-γ) (ng/ml)

The level of serum haptoglobin (µg/ml) indicates a level of response to bacterial inflammation at 24 hours after bacterial challenge (Godson et al., 1995). A one-way ANOVA showed that there was no significant difference between the level of serum haptoglobin (µg/ml) in steers (n=33) and heifers (n=26) with a 2/2 or 2/4 diploid haplotype (P=0.38). Steers and heifers were pooled for further analysis. An unpaired t-test result indicated that there was no significant difference between the level of serum haptoglobin (µg/ml) in cattle with 2/2 and 2/4 diploid haplotypes (P=0.65; Table 5.7).

The peak of virus replication is five days after BHV-1 infection (Raggo et al., 2000) which coincides with the peak of interferon production (Raggo et al., 2000; Hodgson et al., 2012). A one-way ANOVA indicated that there was no significant difference between the level

of interferon-gamma (IFN-γ) (ng/ml) in steers (n=33) and heifers (n=26) with a 2/2 or 2/4 diploid haplotype (*P*=0.30). Steers and heifers were pooled for further analysis. An unpaired t-test result indicated that there was no significant difference between the level of interferon-gamma (IFN-γ) (ng/ml) (Table 5.7) in cattle with 2/2 and 2/4 diploid haplotypes (*P*=0.50; Table 5.7).

Table 5.7: Serum haptoglobin (microgram/ml) and interferon-gamma (IFN-γ) (ng/ml) concentration in 2/2 and 2/4 diploid haplotypes.

Parameter	**Diploid Haplotype**		**P-value**
	2/2 (n=23)	**2/4 (n=30)**	
Serum haptoglobin (µg/ml)	2943 ± 1591.6*	3167.2 ± 1878.8	P=0.65
Interferon-gamma (IFN-γ)(ng/ml)	6363 ± 6661	8085 ± 10623	P=0.50

* mean ± SD

5.4 Discussion

All the animals in these three disease association studies were genotyped for SNPs in the 5′UTR of the *DEFB103* gene by designing a semi-nested PCR (Table 5.1). Four haploid haplotypes (Table 5.2) were determined based on the seven SNPs (c.-383A>G, c.-319A>G, c.-264C>T, c.-241G>A, c.-69A>G, c.-42A>G, and c.-34G>A) in beef and Holstein cattle. The frequency and the number of haplotypes were different in beef and Holstein cattle populations. All the possible haploid haplotypes (n= 4) were identified in Holstein cattle based on the seven SNPs. While only two haploid haplotypes (2 and 4) were identified in Hereford cattle with Cancer-eye and other healthy beef cattle that were analyzed in the Cancer-eye study (Table 5.5). In the viral and bacterial-challenged calves (Angus breed) three haploid haplotypes (1, 2 and 4) were determined, but the frequency of the haploid haplotype 1 in Angus' calves (0.04; Table 5.6) was lower than in Holstein cattle (0.11; Table 5.3).

The Angus samples, collected for the shipping fever study in 2012, were the only beef cattle having haploid haplotype 1. The other samples from a variety of beef cattle breeds including Angus, Hereford, Simmental, and Charolais that were tested for *DEFB103* SNP identification at the early stage of this project were collected in the early 1990s. They did not have haploid haplotype 1. Whereas Holstein samples from the early 1990s in the current mastitis study had haploid haplotype 1. Since selection strategies developed in the beef cattle industry in the past years, the haploid haplotype 1 may have arisen independently by indirect selection in beef cattle over this time.

Although haploid and diploid haplotypes were studied in two Holstein populations regarding mastitis, no significant association was found. The trend toward increased and decreased the risk of mastitis was identified in the two cattle population in this study. The small sample size probably affected the outcome of this study (Table 5.3). The statistical analysis would be more reliable if we had a bigger sample size for each haploid or diploid haplotype.

Antimicrobial peptides affect some mastitis pathogens. *Nocardia* Gram-negative bacteria may cause bovine mastitis (Ribeiro et al., 2008). An *In vitro* study showed, that hBD-3, the human equivalent of cattle DEFB103 had the strongest activity against *N. nova* and *N.farcinica* in comparison to other human antimicrobial peptides (Rieg et al., 2010).

Seven to eight years of age is the peak age of onset for Cancer-eye in cattle (Tsujita and Plummer, 2010). Animals of the available control group in the Cancer-eye study were younger than seven years of age. They were therefore not considered to be an appropriate control for this study. However, all the diploid haplotype combinations (including 2/2, 2/4 and 4/4) were found between Hereford cattle with Cancer-eye and healthy beef cattle. The haplotype frequencies of these two groups of animals had no significant difference (P= 0.43).

The most common site for Cancer-eye development is the nictitating membrane. The current *DEFB103* gene expression study showed a high level of expression in the nictitating membrane in 10-14 day-old calves (Figure 3.2 a; Figure 3.5b). Determining the *DEFB103* gene expression in nictitating membrane tissue of older animals may help us to explain the possible association of this cancer and *DEFB10* expression.

Calves in the shipping fever study were inoculated with *Mannheimia haemolytica*, and bovine herpesvirus-1 (BHV-1). The BHV-1 and the herpes simplex virus (HSV) are members of *Alphaherpesvirinae* subfamily, and they have similar activity and both cause infection in the respiratory tract (Graham and Snell, 1983; Jones, 2003). hBD-3 has been shown to interfere with herpes simplex virus infection in humans (Hazrati et al., 2006). Herpes simplex virus has glycoprotein B and heparan sulfate receptors on its surface. The hBD-3 binds to these two receptors and inhibits infection by preventing HSV binding and entry (Hazrati et al., 2006).

A DNA vaccine that encodes the mature peptide of bovine *β-defensin 3* was designed to protect cattle from bovine herpes virus 1 (Mackenzie-Dyck et al., 2014). The DNA vaccine did not prevent bovine herpes virus 1 infection, but it did improve the cell-mediated immune responses. This result may support the lack of association between the *DEFB103* haplotypes and shipping fever in our study because of vaccine failure, but it does not support the immune response results.

In this study, it has been shown that *DEFB103* complete and partial transcripts have an intron (intron 1a) before the first coding exon (Table 4.3; Figure 4.2). Intron 1a excludes 3 SNPs (c.-319A>G, c.-264C>T, c.-241G>A) from the transcript haplotypes (Table 4.3). Therefore, these three SNPs would not be transcribed in cDNA. The new haploid haplotypes derived from transcripts were called X (equivalent to haploid haplotype 1 and 2), Y (equivalent to haploid

haplotype 3), and Z (equivalent to haploid haplotype 4). Transcripts are the active form of the gene. Therefore, new analyses were done for the mastitis and the shipping fever study using the new diploid haplotypes (X/X, X/Z, and Z/Z), but they did not change the earlier results based on haplotypes that included all 7 SNPs.

Some cattle or calves had two different diploid haplotypes in their incomplete or complete transcripts (Table 4.4). For example, tongue tissue in animal Q has X/X and X/Z diploid haplotypes. Based on this observation, some of the animals in the three studies in this chapter may have more than two diploid haplotypes of *DEFB103* in target tissues, and this may affect the results and their inconsistency.

6.0 GENERAL DISCUSSION

6.1 *DEFB103* Gene Expression

Most diseases are multifactorial in etiology, and they may be controlled by multiple genes, as well as environmental triggers. Therefore, understanding the genetic basis of disease susceptibility may not be as simple as identifying a single gene with a dominant or a recessive effect.

Gene expression results are more accurate when no primer-dimers form or genomic DNA (gDNA) contamination is absent from qRT-PCR reactions. In this situation, cDNA is the only template for the primers (Laurell et al., 2012). Competition of gDNA and cDNA for primers and dNTPs in qRT-PCR may affect the efficiency of the reaction (Laurell et al., 2012). Genomic DNA (gDNA) contamination also may shift C_t values especially in genes with lower gene expression (Yee et al., 2014).

Complementary DNA samples were used for both qRT-PCR (gene expression) and RT-PCR (cDNA haplotype analysis) studies. To minimize the possibility of gDNA contamination, all RNA samples were treated with DNase-I (RNase-free; Invitrogen). Primers were designed that targeted two coding exons with an intervening intron. Furthermore, a melting curve analysis (Appendix D) was performed to determine whether there was a non-specific product or primer dimer formation during the qRT-PCR reactions. All melting curves for each qRT-PCR reaction showed single peaks for *DEFB103* and *B-actin* products and confirmed the absence of gDNA from cDNA.

A gene expression study was conducted for *CBD1*, *CBD103* (*DEFB103* ortholog in dogs) and *CBD108* by Leonard et al. (2012). The *CBD103* gene had a more restricted tissue-specific expression pattern in comparison to *CBD1* and *CBD108*. *CBD103* gene expression was higher in skin and tongue in dog which is consistent with our findings. The highest level of *hBD*-3 expression was also detected in human skin and tonsils (Harder et al., 2001). In the current study, the buccal epithelium was the only tissue that displayed an up-regulation of *DEFB103* following BHV-1 infection in cattle. In general, stratified squamous epithelial tissues had a high level of *DEFB103* expression in comparison to the other tissues.

In vitro treatment of human primary keratinocytes and primary tracheal epithelial cells with heat-inactivated bacteria (*P. aeruginosa ;* 10^8 cells/ ml) or tumor necrosis factor α (TNF-α; 10 ng/ml) up-regulated the level of *hBD*-3 expression (Harder et al., 2001). In the current study, the buccal epithelium was the only tissue sampled that significantly up-regulated *DEFB103* gene expression following BHV-1 infection. This observation supports the conclusion that *DEFB103* gene expression can also be modulated by infectious agents. In this situation, other infection disease studies and their relation to *DEFB103* gene expression in a target tissue might be useful. Further study of the effect of mastitis on *DEFB103* gene expression in the mammary gland might be important to clarify results of my other studies in Chapter 5. If *DEFB103* gene expression does not change in the mammary gland during mastitis, then this would be consistent with the results reported for *DEFB103* haplotype study (Chapter 5). *DEFB103* gene expression data may also indicate whether mastitis was an appropriate disease to look for correlation with *DEFB103* haplotypes.

Immunohistochemistry studies confirmed keratinocytes were the main cell type expressing *DEFB103* in the skin of both humans (Harder et al., 2001) and dogs (Leonard et al.,

2012). These observations are in agreement with our findings in cattle. , These previous studies did not report localized production of DEFB103 protein in basal epithelial cells, as we observed in cattle. Some dogs have a 3-bp deletion (K^B allele) in the second exon of *CBD10*3 that removes a glycine from the amino acid sequence. Dogs with this K^B allele have significantly (P = 0.0021) increased CBD103 protein levels in their keratinocytes when compared to dogs homozygous for the wild-type alleles (k^y/k^y) (Candille et al., 2007).

We also observed DEFB103 protein was abundant in the conjunctiva of the bovine eye. A high level of protein production in this tissue indicates that ocular diseases may be appropriate targets to study when investigating potential associations between DEFB103 alleles and disease resistance.

6.2 *DEFB103* Gene Structure and Its Association with Diseases

The *β-defensin* gene cluster contributes to multiple aspects of the innate immune responses, and it may influence the development of infectious disease during the initial steps of infection that occur within epithelial tissues. In humans, the cluster of *β-defensin* genes on chromosome 8 has 2-12 copies per diploid genome (Hollox et al., 2003). The Btau 4.6.1 and UMD 3.1 assemblies verified there were multiple copies of the *DEFB103* gene in cattle (Chapter 4.5.3).

The number of copies of *DEFB103* transcripts (mRNA) was determined based on sequence analysis of RT-PCR products extracted from an agarose gel. In this study, at least, two complete transcripts of the bovine *DEFB103* with different haplotypes were identified for some tissues when using RT-PCR (Chapter 4, Table 4.4). At least two complete copies of *DEFB103* transcripts were identified but because of the RT-PCR method used, I cannot conclude whether these two complete transcripts were generated from two or more gene copies present in the

genome. If the *DEFB103* copies have the same haplotype, then it is not possible to distinguish between the two copies using RT-PCR. This is a limitation of RT-PCR. Therefore, the number of complete transcripts might be more than two copies in some cattle. A copy number variation (CNV) study is needed to determine a minimum and a maximum number of *DEFB103* copies in cattle.

Using manual annotation, Amid et al. (2009) reported 53 active and 22 pseudogenes (5 partial) in the major defensin gene cluster region of mouse chromosome 8. Defensin's pseudogenes in mice are not transcribed, so they are not functional (Amid et al., 2009). Another example is the neutrophil cytosolic factor 1 (*NCF1*) gene on chromosome 7 in human. The *NCF1* has segmental duplication (Antonell et al., 2005) and two of the highly similar copies in *NCF1* do not produce functional protein because of a 2-bp deletion in exon 2 (Gorlach et al., 1997).

All the sequences analyzed from this RT-PCR experiment had ATG as the start codon and were identical except for the seven SNPs positions. *DEFB103* SNPs are present before the start codon. Therefore, we suggest that these two complete transcripts were functional copies of the *DEFB103* gene. A more detailed DEFB103 protein expression and function study are necessary to confirm conclusively that both mRNAs are translated into functional proteins.

The initial assumption for the *DEFB103* gene expression analyses was that all *DEFB103* transcripts were derived from a single copy of the DEFB103 gene. But RT-PCR revealed that this assumption was not valid. Because at least two complete copies of *DEFB103* have been identified in some cattle (Chapter 4, Table 4). I could not include a full characterization of transcript copies in the gene expression analysis because there was insufficient sample for each age category and we did not determine the actual number of transcript copies present in each

sample. If adequate samples were available for each haplotype and different CNVs, the gene expression study would have been more informative.

Genotyping for a gene like *DEFB103* with CNV and pseudogenes is complicated. In each PCR reaction, one copy of a functional gene or a pseudogene could be amplified predominantly and, therefore, SNP genotypes or haplotypes may not be consistent when performing replicate assays. The chance of amplifying a pseudogene is higher if a one-step PCR, rather than a nested PCR, is used for genotyping since both complete and partial copies of the gene could be amplified in this reaction. In this study, all animals were genotyped for seven SNPs in the 5′UTR of *DEFB103* using nested-PCR (explained in Section 4.3.4; Table 4.1) to exclude partial pseudogenes and minimize non-specific amplification. Only complete gene copies were amplified by the first PCR. The nested PCR then amplified a smaller gene fragment (about 700 bp) that was more appropriate for sequencing.

Complementary DNA samples were used for the RT-PCR with the assumption that no gDNA contamination was present. However, at the beginning of this study two PCR products (573 bp including intron 1a and 312 bp with intron 1a spliced out) were amplified using KNEST1-F and DEFEX1-R primers (Table 4.1; Figure 4.1). Multiple products in RT-PCR could be the result of gDNA contamination. Therefore, KNEST1-F and DEFB103EX2-R2 primers were used to amplify the complete transcript that includes two coding exons and non-coding exon 1a (Figure 4.1b). The complete transcript (511 bp; Figure 4.1b) in all samples had non-coding exon 1a and intron 1a and no gDNA was amplified (Appendix C).

Four haploid haplotypes and 10 possible diploid haplotypes were identified in beef and Holstein cattle, respectively. Since the number of diploid haplotypes was 10, we did not have sufficient samples size for each diploid haplotype to analyze them separately in the mastitis

study. This limited the statistical power of our analysis. No significant association was found between the *DEFB103* haplotypes and mastitis, cancer-eye and shipping fever in this study. Including a consideration of CNV and increasing the number of animals sampled may yield different results in a future study.

Wagter and Mallard (2007) patented a test which can identify animals with higher cell-mediated (CMIR) and antibody-mediated immune responses (AMIR). Thompson et al. (2012) reported a lower frequency of infectious and metabolic diseases in Holstein cows with high AMIR and High CMIR. Cows with higher AMIR also had a lower incidence of mastitis (Wagter et al., 2000; Thompson-Crispi et al., 2012). A genome-wide association study of immune response trait revealed a significant association between many genes known to be involved in immune response, such as bovine Major Histocompatibility Complex, cytokines, and complement system with both high AMIR and CMIR and *TAP* and *DEFB103* were associated with high CMIR (Thompson-Crispi et al., 2014c). These findings support the conclusion that animal selection based on an individual's immune responses or genetic profile could improve animal health, decrease the cost of treating disease, and increase the profitability of the dairy and beef cattle industry.

6.3 Future Research

In this study, RT-PCR was used to determine the start and end points of DEFB103 cDNA. Three forward primers were designed from 678 bp to 1624 bp beyond the start codon, but no fragment was amplified. Therefore, I concluded that the cDNA start point was at least 514 bp (KNEST1-F primer position that amplified a fragment) before the start codon and not further than 678 bp. The non-coding exon 1a (Figure 4.2b) identified in the 5′ UTR of the *DEFB103* gene was identified in a later experiment. Identification of the non-coding exon 1a in the 5′ UTR

indicated the possible presence of one or more non-coding exons beyond the 514 bp upstream of the start codon. Therefore, a more detailed study may be needed to determine accurately the start point of the 5′ UTR of the *DEFB103* gene. The length of the identified non-coding exon 1a based on these experiments is, at least, 383bp and not larger than 556 bp.

The presence of non-coding exon 1a was predicted in sheep and goat based on BLAST analysis. An experiment could be conducted to verify the presence of the non-coding exon 1a in sheep and goat. If the non-coding exon 1a is also present in sheep and goat, this would indicate that the *DEFB103* gene structure is conserved in ruminants and differentiate it from other species.

The *β-defensin* gene family members in the BTA27 cluster have multiple copy numbers or CNV, and it has been reported in Chapter 4 that some cattle had more than one haplotype based on the repeated PCR reactions in *DEFB103* (Table 4.4). Copy number variation for *DEFB103* among individual animals may have affected the disease association analyses in this study. There may be a possible significant association between infectious disease susceptibility and *DEFB103* gene variation in copy number, but this possible association will need to be explored in future studies

The analysis of *DEFB103* gene haplotypes and SNPs did not reveal any significant associations with the three diseases studied (Chapter 5). The *DEFB103* gene is a member of a *β-defensin* gene family, and this gene family is one of the primary gene clusters in the innate immunity system. Other *β-defensin* gene family members could be investigated for potential associations with the infectious diseases that I studied. One of the candidate genes for mastitis disease might be the *β-defensin 5*. Goldammer et al. (2004) reported *β-defensin 5* gene up-regulation during mastitis in cattle. LAP protein has also been identified in infected and healthy

mammary gland of cattle (Isobe et al., 2009) and its expression in cattle had a positive relationship with somatic cell count in milk (Swanson et al., 2004).

6.4 Conclusion

- *DEFB103* gene expression and protein was significantly higher in the oral cavity and stratified squamous epithelium of skin in comparison to the other tissues collected from 14-day-old calves (Figure 3.2a).
- Buccal epithelium was the only tissue in which *DEFB103* gene expression was up-regulated following infection or down-regulated with increasing age. *DEFB103* gene expression was significantly increased ($p<0.05$) in the buccal epithelium of infected 6- to 8-month old animals in comparison to healthy 8- to 10-month old animals (Figure 3.4b). *DEFB103* gene expression was significantly decreased ($p<0.05$) in the buccal epithelium of 10- to 12-month old animals in comparison to 10- to 14-day old calves (Figure 3.3).
- A new non-coding exon 1a was reported in the 5′ UTR of the *DEFB103* gene. This genomic structure has not been reported in any other *β-defensin* genes studied in other species. Two new SNPs were also identified in the 5′ UTR of the *DEFB103* gene. The combination of two new SNPs and the five previously reported SNPs (Dreger and Schmutz, 2009) identified two new haplotypes for *DEFB103* gene.
- In this study, no significant association was found between the *DEFB103* haplotypes and mastitis, cancer-eye or fetal secondary bacterial respiratory disease following a primary BHV-1 infection.

7.0 LITERATURE CITED

Abdel-Shafy, H., Bortfeldt, R.H., Tetens, J. and Brockmann, G.A. Single nucleotide polymorphism and haplotype effects associated with somatic cell score in German Holstein cattle. *Genet Sel Evol* **46** (2014), p. 35.

Agerberth, B. and Gudmundsson, G.H. Host antimicrobial defence peptides in human disease. *Curr Top Microbiol Immunol* **306** (2006), pp. 67-90.

Alkan, C., Coe, B.P. and Eichler, E.E. Genome structural variation discovery and genotyping. *Nat Rev Genet* **12** (2011), pp. 363-76.

Almal, S.H. and Padh, H. Implications of gene copy-number variation in health and diseases. *J Hum Genet* **57** (2012), pp. 6-13.

Amae, S., Fuse, N., Yasumoto, K., Sato, S., Yajima, I., Yamamoto, H., Udono, T., Durlu, Y.K., Tamai, M., Takahashi, K. and Shibahara, S. Identification of a novel isoform of microphthalmia-associated transcription factor that is enriched in retinal pigment epithelium. *Biochem Biophys Res Commun* **247** (1998), pp. 710-5.

Amarger, V., Nguyen, M., Van Laere, A.S., Braunschweig, M., Nezer, C., Georges, M. and Andersson, L. Comparative sequence analysis of the INS-IGF2-H19 gene cluster in pigs. *Mamm Genome* **13** (2002), pp. 388-98.

Amid, C., Rehaume, L.M., Brown, K.L., Gilbert, J.G., Dougan, G., Hancock, R.E. and Harrow, J.L. Manual annotation and analysis of the defensin gene cluster in the C57BL/6J mouse reference genome. *BMC Genomics* **10** (2009), p. 606.

Antonell, A., de Luis, O., Domingo-Roura, X. and PÃ©rez-Jurado, L.A. Evolutionary mechanisms shaping the genomic structure of the Williams-Beuren syndrome chromosomal region at human 7q11.23. *Genome Research* **15** (2005), pp. 1179-1188.

Apley, M. The clinical syndrome of BRD: what it is and what it is not. *Anim Health Res Rev* **15** (2014), pp. 135-7.

Bagnicka, E., Strzalkowska, N., Jozwik, A., Krzyzewski, J., Horbanczuk, J. and Zwierzchowski, L. Expression and polymorphism of defensins in farm animals. *Acta Biochim Pol* **57** (2010), pp. 487-97.

Balakirev, E.S. and Ayala, F.J. PSEUDOGENES: Are They "Junk" or Functional DNA? *Annual Review of Genetics* **37** (2003), pp. 123-151.

Bentley, R.W., Pearson, J., Gearry, R.B., Barclay, M.L., McKinney, C., Merriman, T.R. and Roberts, R.L. Association of higher DEFB4 genomic copy number with Crohn's disease. *Am J Gastroenterol* **105** (2010), pp. 354-9.

Bevins, C.L. Paneth cell defensins: key effector molecules of innate immunity. *Biochem Soc Trans* **34** (2006), pp. 263-6.

Bevins, C.L., Jones, D.E., Dutra, A., Schaffzin, J. and Muenke, M. Human enteric defensin genes: chromosomal map position and a model for possible evolutionary relationships. *Genomics* **31** (1996), pp. 95-106.

Blackwood, E.M. and Kadonaga, J.T. Going the distance: a current view of enhancer action. *Science* **281** (1998), pp. 60-3.

Boisvert, F.M., Ahmad, Y., Gierlinski, M., Charriere, F., Lamont, D., Scott, M., Barton, G. and Lamond, A.I. A quantitative spatial proteomics analysis of proteome turnover in human cells. *Mol Cell Proteomics* **11** (2012), p. M111 011429.

Boman, H.G. Peptide antibiotics and their role in innate immunity. *Annu Rev Immunol* **13** (1995), pp. 61-92.

Bonilla, F.A. and Oettgen, H.C. Adaptive immunity. *Journal of Allergy and Clinical Immunology* **125** (2010), pp. S33-S40.

Box, N.F., Wyeth, J.R., Mayne, C.J., O'Gorman, L.E., Martin, N.G. and Sturm, R.A. Complete sequence and polymorphism study of the human TYRP1 gene encoding tyrosinase-related protein 1. *Mamm Genome* **9** (1998), pp. 50-3.

Bruder, C.E., Piotrowski, A., Gijsbers, A.A., Andersson, R., Erickson, S., Diaz de Stahl, T., Menzel, U., Sandgren, J., von Tell, D., Poplawski, A., Crowley, M., Crasto, C., Partridge, E.C., Tiwari, H., Allison, D.B., Komorowski, J., van Ommen, G.J., Boomsma, D.I., Pedersen, N.L., den Dunnen, J.T., Wirdefeldt, K. and Dumanski, J.P. Phenotypically concordant and discordant monozygotic twins display different DNA copy-number-variation profiles. *Am J Hum Genet* **82** (2008), pp. 763-71.

Bruhn, O., Regenhard, P., Michalek, M., Paul, S., Gelhaus, C., Jung, S., Thaller, G., Podschun, R., Leippe, M., Grotzinger, J. and Kalm, E. A novel horse alpha-defensin: gene

transcription, recombinant expression and characterization of the structure and function. *Biochem J* **407** (2007), pp. 267-76.

Bryan, L.A., Griebel, P.J., Haines, D.M., Davis, W.C. and Allen, J.R. Immunocytochemical identification of bovine Langerhans cells by use of a monoclonal antibody directed against class II MHC antigens. *Journal of Histochemistry & Cytochemistry* **36** (1988), pp. 991-5.

Buchanan, F.C., Fitzsimmons, C.J., Van Kessel, A.G., Thue, T.D., Winkelman-Sim, D.C. and Schmutz, S.M. Association of a missense mutation in the bovine leptin gene with carcass fat content and leptin mRNA levels. *Genet Sel Evol* **34** (2002), pp. 105-16.

Candille, S.I., Kaelin, C.B., Cattanach, B.M., Yu, B., Thompson, D.A., Nix, M.A., Kerns, J.A., Schmutz, S.M., Millhauser, G.L. and Barsh, G.S. A Beta-defensin mutation causes black coat color in domestic dogs. *Science* **318** (2007), pp. 1418-23.

Carninci, P., Sandelin, A., Lenhard, B., Katayama, S., Shimokawa, K., Ponjavic, J., Semple, C.A.M., Taylor, M.S., Engstrom, P.G., Frith, M.C., Forrest, A.R.R., Alkema, W.B., Tan, S.L., Plessy, C., Kodzius, R., Ravasi, T., Kasukawa, T., Fukuda, S., Kanamori-Katayama, M., Kitazume, Y., Kawaji, H., Kai, C., Nakamura, M., Konno, H., Nakano, K., Mottagui-Tabar, S., Arner, P., Chesi, A., Gustincich, S., Persichetti, F., Suzuki, H., Grimmond, S.M., Wells, C.A., Orlando, V., Wahlestedt, C., Liu, E.T., Harbers, M., Kawai, J., Bajic, V.B., Hume, D.A. and Hayashizaki, Y. Genome-wide analysis of mammalian promoter architecture and evolution. *Nat Genet* **38** (2006), pp. 626-635.

Cederlund, A., Gudmundsson, G.H. and Agerberth, B. Antimicrobial peptides important in innate immunity. *FEBS J* **278** (2011), pp. 3942-51.

Charavaryamath, C., Fries, P., Gomis, S., Bell, C., Doig, K., Guan, L.L., Potter, A., Napper, S. and Griebel, P.J. Mucosal changes in a long-term bovine intestinal segment model following removal of ingesta and microflora. *Gut Microbes* **2** (2011), pp. 134-44.

Cormican, P., Meade, K., Cahalane, S., Narciandi, F., Chapwanya, A., Lloyd, A. and O' Farrelly, C. Evolution, expression and effectiveness in a cluster of novel bovine Beta-defensins. *Immunogenetics* **60** (2008), pp. 147-156.

Davila Lopez, M., Martinez Guerra, J.J. and Samuelsson, T. Analysis of gene order conservation in eukaryotes identifies transcriptionally and functionally linked genes. *PLoS One* **5** (2010), p. e10654.

De Conti, L., Baralle, M. and Buratti, E. Exon and intron definition in pre-mRNA splicing. *Wiley Interdiscip Rev RNA* **4** (2013), pp. 49-60.

de Vooght, K.M.K., van Wijk, R. and van Solinge, W.W. Management of Gene Promoter Mutations in Molecular Diagnostics. *Clinical Chemistry* **55** (2009), pp. 698-708.

Diamond, G., Kaiser, V., Rhodes, J., Russell, J.P. and Bevins, C.L. Transcriptional Regulation of B-Defensin Gene Expression in Tracheal Epithelial Cells. *Infection and Immunity* **68** (2000), pp. 113-119.

Diamond, G. and Ryan, L. Beta-defensins: what are they really doing in the oral cavity? *Oral Dis* **17** (2011), pp. 628-35.

Diamond, G., Zasloff, M., Eck, H., Brasseur, M., Maloy, W.L. and Bevins, C.L. Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. *Proc Natl Acad Sci U S A* **88** (1991), pp. 3952-6.

Dickinson, D.P., Ridall, A.L. and Levine, M.J. Human submandibular gland statherin and basic histidine-rich peptide are encoded by highly abundant mRNA's derived from a common ancestral sequence. *Biochem Biophys Res Commun* **149** (1987), pp. 784-90.

Dreger, D.L. and Schmutz, S.M. The variant red coat colour phenotype of Holstein cattle maps to BTA27. *Anim Genet* **41** (2009), pp. 109-12.

Eisenberg, E. and Levanon, E.Y. Human housekeeping genes, revisited. *Trends Genet* **29** (2013), pp. 569-74.

Elsik, C.G., Tellam, R.L., Worley, K.C., Gibbs, R.A., Muzny, D.M., Weinstock, G.M., Adelson, D.L., Eichler, E.E., Elnitski, L. and Guigo, R. The genome sequence of taurine cattle: a window to ruminant biology and evolution. *Science* **324** (2009), pp. 522-8.

Fadista, J., Thomsen, B., Holm, L.E. and Bendixen, C. Copy number variation in the bovine genome. *BMC Genomics* **11** (2010), p. 284.

Fellermann, K., Stange, D.E., Schaeffeler, E., Schmalzl, H., Wehkamp, J., Bevins, C.L., Reinisch, W., Teml, A., Schwab, M., Lichter, P., Radlwimmer, B. and Stange, E.F. A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. *Am J Hum Genet* **79** (2006), pp. 439-48.

Feuk, L., Carson, A.R. and Scherer, S.W. Structural variation in the human genome. *Nat Rev Genet* **7** (2006), pp. 85-97.

Fjell, C.D., Jenssen, H., Fries, P., Aich, P., Griebel, P., Hilpert, K., Hancock, R.E. and Cherkasov, A. Identification of novel host defense peptides and the absence of alpha-defensins in the bovine genome. *Proteins* **73** (2008), pp. 420-30.

Flanigan, K.M., Dunn, D.M., von Niederhausern, A., Soltanzadeh, P., Howard, M.T., Sampson, J.B., Swoboda, K.J., Bromberg, M.B., Mendell, J.R., Taylor, L., Anderson, C.B., Pestronk, A., Florence, J., Connolly, A.M., Mathews, K.D., Wong, B., Finkel, R.S., Bonnemann, C.G., Day, J.W., McDonald, C., the United Dystrophinopathy Project, C. and Weiss, R.B. Nonsense mutation-associated Becker muscular dystrophy: interplay between exon definition and splicing regulatory elements within the DMD gene. *Human Mutation* **32** (2011), pp. 299-308.

Fode, P., Stegger, M. and Andersen, P.S. Human beta-defensin 3 (DEFB103) and its influence on Staphylococcus aureus nasal carriage. *Int J Infect Dis* **15** (2011), pp. e388-94.

Fox, L.K. Mycoplasma Mastitis: Causes, Transmission, and Control. *Veterinary Clinics of North America: Food Animal Practice* **28** (2012), pp. 225-237.

Fries, P., Popowych, Y.I., Guan, L.L., Beskorwayne, T., Potter, A., Babiuk, L. and Griebel, P.J. Mucosal dendritic cell subpopulations in the small intestine of newborn calves. *Dev Comp Immunol* **35** (2011), pp. 1040-51.

Funderburg, N., Lederman, M.M., Feng, Z., Drage, M.G., Jadlowsky, J., Harding, C.V., Weinberg, A. and Sieg, S.F. Human -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. *Proc Natl Acad Sci U S A* **104** (2007), pp. 18631-5.

Gallagher, D.S., Jr., Ryan, A.M., Diamond, G., Bevins, C.L. and Womack, J.E. Somatic cell mapping of beta-defensin genes to cattle syntenic group U25 and fluorescence in situ localization to chromosome 27. *Mamm Genome* **6** (1995), pp. 554-6.

Ganz, T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* **3** (2003a), pp. 710-20.

Ganz, T. The role of antimicrobial peptides in innate immunity. *Integr Comp Biol* **43** (2003b), pp. 300-4.

Ganz, T. and Lehrer, R.I. Defensins. *Curr Opin Immunol* **6** (1994), pp. 584-9.

Ganz, T. and Lehrer, R.I. Defensins. *Pharmacol Ther* **66** (1995), pp. 191-205.

Ganz, T., Selsted, M.E., Szklarek, D., Harwig, S.S., Daher, K., Bainton, D.F. and Lehrer, R.I. Defensins. Natural peptide antibiotics of human neutrophils. *J Clin Invest* **76** (1985), pp. 1427-35.

Garcia, J.R., Jaumann, F., Schulz, S., Krause, A., Rodriguez-Jimenez, J., Forssmann, U., Adermann, K., Kluver, E., Vogelmeier, C., Becker, D., Hedrich, R., Forssmann, W.G. and Bals, R. Identification of a novel, multifunctional beta-defensin (human beta-defensin 3) with specific antimicrobial activity. Its interaction with plasma membranes of Xenopus oocytes and the induction of macrophage chemoattraction. *Cell Tissue Res* **306** (2001), pp. 257-64.

Gardiner-Garden, M. and Frommer, M. CpG Islands in vertebrate genomes. *Journal of Molecular Biology* **196** (1987), pp. 261-282.

Gilbert, W. Why genes in pieces? *Nature* **271** (1978), p. 501.

Goding, C.R. Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage. *Genes Dev* **14** (2000), pp. 1712-28.

Godson, D.L., Baca-Estrada, M.E., Van Kessel, A.G., Hughes, H.P., Morsy, M.A., Van Donkersgoed, J., Harland, R.J., Shuster, D.E., Daley, M.J. and Babiuk, L.A. Regulation of bovine acute phase responses by recombinant interleukin-1 beta. *Can J Vet Res* **59** (1995), pp. 249-55.

Goldammer, T., Zerbe, H., Molenaar, A., Schuberth, H.J., Brunner, R.M., Kata, S.R. and Seyfert, H.M. Mastitis increases mammary mRNA abundance of beta-defensin 5, toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. *Clin Diagn Lab Immunol* **11** (2004), pp. 174-85.

Gonzalez-Cano, P., Arsic, N., Popowych, Y.I. and Griebel, P.J. Two functionally distinct myeloid dendritic cell subpopulations are present in bovine blood. *Dev Comp Immunol* **44** (2014), pp. 378-88.

Goodall, J.J. and Schmutz, S.M. IGF2 gene characterization and association with rib eye area in beef cattle. *Anim Genet* **38** (2007), pp. 154-61.

Goren, A., Ram, O., Amit, M., Keren, H., Lev-Maor, G., Vig, I., Pupko, T. and Ast, G. Comparative analysis identifies exonic splicing regulatory sequences:The complex definition of enhancers and silencers. *Mol Cell* **22** (2006), pp. 769-81.

Gorlach, A., Lee, P.L., Roesler, J., Hopkins, P.J., Christensen, B., Green, E.D., Chanock, S.J. and Curnutte, J.T. A p47-phox pseudogene carries the most common mutation causing p47-phox- deficient chronic granulomatous disease. *Journal of Clinical Investigation* **100** (1997), pp. 1907-1918.

Graham, B.S. and Snell, J.D., Jr. Herpes simplex virus infection of the adult lower respiratory tract. *Medicine (Baltimore)* **62** (1983), pp. 384-93.

Griebel, P., Hill, K. and Stookey, J. How stress alters immune responses during respiratory infection. *Animal Health Research Reviews* **15** (2014), pp. 161-165.

Griebel, P.J., Kennedy, L., Graham, T., Davis, W.C. and Reynolds, J.D. Characterization of B-cell phenotypic changes during ileal and jejunal Peyer's patch development in sheep. *Immunology* **77** (1992), pp. 564-70.

Haase, B., Doherr, M.G., Seuberlich, T., Drogemuller, C., Dolf, G., Nicken, P., Schiebel, K., Ziegler, U., Groschup, M.H., Zurbriggen, A. and Leeb, T. PRNP promoter polymorphisms are associated with BSE susceptibility in Swiss and German cattle. *BMC Genet* **8** (2007), p. 15.

Hammad, H. and Lambrecht, B.N. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* **8** (2008), pp. 193-204.

Han, J., Xiong, J., Wang, D. and Fu, X.-D. Pre-mRNA splicing: where and when in the nucleus. *Trends in Cell Biology* **21** (2011), pp. 336-343.

HapMap The International HapMap Project. *Nature* **426** (2003), pp. 789-796.

Harder, J., Bartels, J., Christophers, E. and Schroder, J.M. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* **276** (2001), pp. 5707-13.

Hardwick, R.J., Machado, L.R., Zuccherato, L.W., Antolinos, S., Xue, Y., Shawa, N., Gilman, R.H., Cabrera, L., Berg, D.E., Tyler-Smith, C., Kelly, P., Tarazona-Santos, E. and Hollox, E.J. A worldwide analysis of beta-defensin copy number variation suggests recent selection of a high-expressing DEFB103 gene copy in East Asia. *Hum Mutat* **32** (2011), pp. 743-50.

Harvey, L.E., Kohlgraf, K.G., Mehalick, L.A., Raina, M., Recker, E.N., Radhakrishnan, S., Prasad, S.A., Vidva, R., Progulske-Fox, A., Cavanaugh, J.E., Vali, S. and Brogden, K.A.

Defensin DEFB103 bidirectionally regulates chemokine and cytokine responses to a pro-inflammatory stimulus. *Sci Rep* **3** (2013), p. 1232.

Hastings, P.J., Lupski, J.R., Rosenberg, S.M. and Ira, G. Mechanisms of change in gene copy number. *Nat Rev Genet* **10** (2009), pp. 551-64.

Hazlett, L. and Wu, M. Defensins in innate immunity. *Cell Tissue Res* **343** (2011), pp. 175-88.

Hazrati, E., Galen, B., Lu, W., Wang, W., Ouyang, Y., Keller, M.J., Lehrer, R.I. and Herold, B.C. Human alpha and B-Defensins Block Multiple Steps in Herpes Simplex Virus Infection. *The Journal of Immunology* **177** (2006), pp. 8658-8666.

Henrichsen, C.N., Chaignat, E. and Reymond, A. Copy number variants, diseases and gene expression. *Hum Mol Genet* **18** (2009), pp. R1-8.

Hesketh, J.: 3′ UTRs and Regulation, *eLS*. John Wiley & Sons, Ltd (2001).

Hilton, W.M. BRD in 2014: where have we been, where are we now, and where do we want to go? *Anim Health Res Rev* **15** (2014), pp. 120-2.

Hodgson, P.D., Aich, P., Stookey, J., Popowych, Y., Potter, A., Babiuk, L. and Griebel, P.J. Stress significantly increases mortality following a secondary bacterial respiratory infection. *Vet Res* **43** (2012), p. 21.

Hollox, E.J. Copy number variation of beta-defensins and relevance to disease. *Cytogenet Genome Res* **123** (2008), pp. 148-55.

Hollox, E.J., Armour, J.A. and Barber, J.C. Extensive normal copy number variation of a beta-defensin antimicrobial-gene cluster. *Am J Hum Genet* **73** (2003), pp. 591-600.

Hollox, E.J., Huffmeier, U., Zeeuwen, P.L., Palla, R., Lascorz, J., Rodijk-Olthuis, D., van de Kerkhof, P.C., Traupe, H., de Jongh, G., den Heijer, M., Reis, A., Armour, J.A. and Schalkwijk, J. Psoriasis is associated with increased beta-defensin genomic copy number. *Nat Genet* **40** (2008), pp. 23-5.

Holthuizen, P.E., Cleutjens, C.B., Veenstra, G.J., van der Lee, F.M., Koonen-Reemst, A.M. and Sussenbach, J.S. Differential expression of the human, mouse and rat IGF-II genes. *Regul Pept* **48** (1993), pp. 77-89.

Hou, Y., Bickhart, D.M., Hvinden, M.L., Li, C., Song, J., Boichard, D.A., Fritz, S., Eggen, A., DeNise, S., Wiggans, G.R., Sonstegard, T.S., Van Tassell, C.P. and Liu, G.E. Fine mapping of copy number variations on two cattle genome assemblies using high density SNP array. *BMC Genomics* **13** (2012), p. 376.

Hou, Y., Liu, G.E., Bickhart, D.M., Cardone, M.F., Wang, K., Kim, E.S., Matukumalli, L.K., Ventura, M., Song, J., VanRaden, P.M., Sonstegard, T.S. and Van Tassell, C.P. Genomic characteristics of cattle copy number variations. *BMC Genomics* **12** (2011), p. 127.

Huang, Y.Z., Zhan, Z.Y., Li, X.Y., Wu, S.R., Sun, Y.J., Xue, J., Lan, X.Y., Lei, C.Z., Zhang, C.L., Jia, Y.T. and Chen, H. SNP and haplotype analysis reveal IGF2 variants associated with growth traits in Chinese Qinchuan cattle. *Mol Biol Rep* **41** (2014), pp. 591-8.

Ishimoto, H., Mukae, H., Date, Y., Shimbara, T., Mondal, M.S., Ashitani, J., Hiratsuka, T., Kubo, S., Kohno, S. and Nakazato, M. Identification of hBD-3 in respiratory tract and serum: the increase in pneumonia. *Eur Respir J* **27** (2006), pp. 253-60.

Isobe, N., Hosoda, K. and Yoshimura, Y. Immunolocalization of lingual antimicrobial peptide (LAP) in the bovine mammary gland. *Anim Sci J* **80** (2009), pp. 446-50.

Jacox, E., Gotea, V., Ovcharenko, I. and Elnitski, L. Tissue-specific and ubiquitous expression patterns from alternative promoters of human genes. *PLoS One* **5** (2010), p. e12274.

Jarczak, J., Kosciuczuk, E.M., Lisowski, P., Strzalkowska, N., Jozwik, A., Horbanczuk, J., Krzyzewski, J., Zwierzchowski, L. and Bagnicka, E. Defensins: natural component of human innate immunity. *Hum Immunol* **74** (2013), pp. 1069-79.

Jones, C. Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Microbiol Rev* **16** (2003), pp. 79-95.

Kabelitz, D. and Medzhitov, R. Innate immunity--cross-talk with adaptive immunity through pattern recognition receptors and cytokines. *Curr Opin Immunol* **19** (2007), pp. 1-3.

Kallioniemi, A., Kallioniemi, O.P., Sudar, D., Rutovitz, D., Gray, J.W., Waldman, F. and Pinkel, D. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* **258** (1992), pp. 818-21.

Kimbrell, D.A. and Beutler, B. The evolution and genetics of innate immunity. *Nat Rev Genet* **2** (2001), pp. 256-267.

Komar, A.A. Genetics. SNPs, silent but not invisible. *Science* **315** (2007), pp. 466-7.

Lai, Y. and Gallo, R.L. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends in Immunology* **30** (2009), pp. 131-141.

Lang, T.A. and Secic, M., How to report statistic in medicine, annotated guidelines for authors, editors, and reviewrs. , second ed. binding by Sheridan Press., USA (2006).

Laurell, H., Iacovoni, J.S., Abot, A., Svec, D., Maoret, J.-J., Arnal, J.-F.o. and Kubista, M. Correction of RT-qPCR data for genomic DNA-derived signals with ValidPrime. *Nucleic Acids Research* (2012).

Lehrer, R.I. Primate defensins. *Nat Rev Microbiol* **2** (2004), pp. 727-38.

Lehrer, R.I. and Ganz, T. Defensins: endogenous antibiotic peptides from human leukocytes. *Ciba Found Symp* **171** (1992), pp. 276-90; discussion 290-3.

Lehrer, R.I. and Ganz, T. Antimicrobial peptides in mammalian and insect host defence. *Curr Opin Immunol* **11** (1999), pp. 23-7.

Lehrer, R.I. and Ganz, T. Cathelicidins: a family of endogenous antimicrobial peptides. *Curr Opin Hematol* **9** (2002), pp. 18-22.

Leipprandt, J.R., Chen, H., Horvath, J.E., Qiao, X.T., Jones, M.Z. and Friderici, K.H. Identification of a bovine Î²-mannosidosis mutation and detection of two Î²-mannosidase pseudogenes. *Mammalian Genome* **10** (1999), pp. 1137-1141.

Leonard, B.C., Marks, S.L., Outerbridge, C.A., Affolter, V.K., Kananurak, A., Young, A., Moore, P.F., Bannasch, D.L. and Bevins, C.L. Activity, expression and genetic variation of canine beta-defensin 103: a multifunctional antimicrobial peptide in the skin of domestic dogs. *J Innate Immun* **4** (2012), pp. 248-59.

Levin, C., Perrin, H. and Combadiere, B. Tailored immunity by skin antigen-presenting cells. *Human Vaccines & Immunotherapeutics* **11** (2014), p. e34299.

Li, R.W. and Gasbarre, L.C. A temporal shift in regulatory networks and pathways in the bovine small intestine during Cooperia oncophora infection. *Int J Parasitol* **39** (2009), pp. 813-24.

Li, X.-Q. and Du, D. Gene direction in living organisms. *Sci. Rep.* **2** (2012).

Liang, G., Malmuthuge, N., McFadden, T.B., Bao, H., Griebel, P.J., Stothard, P. and Guan le, L. Potential regulatory role of microRNAs in the development of bovine gastrointestinal tract during early life. *PLoS One* **9** (2014), p. e92592.

Liu, G.E., Brown, T., Hebert, D.A., Cardone, M.F., Hou, Y., Choudhary, R.K., Shaffer, J., Amazu, C., Connor, E.E., Ventura, M. and Gasbarre, L.C. Initial analysis of copy number variations in cattle selected for resistance or susceptibility to intestinal nematodes. *Mamm Genome* **22** (2011), pp. 111-21.

Liu, G.E., Hou, Y., Zhu, B., Cardone, M.F., Jiang, L., Cellamare, A., Mitra, A., Alexander, L.J., Coutinho, L.L., Dell'Aquila, M.E., Gasbarre, L.C., Lacalandra, G., Li, R.W., Matukumalli, L.K., Nonneman, D., Regitano, L.C., Smith, T.P., Song, J., Sonstegard, T.S., Van Tassell, C.P., Ventura, M., Eichler, E.E., McDaneld, T.G. and Keele, J.W. Analysis of copy number variations among diverse cattle breeds. *Genome Res* **20** (2010), pp. 693-703.

Liu, G.E., Van Tassel, C.P., Sonstegard, T.S., Li, R.W., Alexander, L.J., Keele, J.W., Matukumalli, L.K., Smith, T.P. and Gasbarre, L.C. Detection of germline and somatic copy number variations in cattle. *Dev Biol (Basel)* **132** (2008), pp. 231-7.

Liu, L., Zhao, C., Heng, H.H. and Ganz, T. The human beta-defensin-1 and alpha-defensins are encoded by adjacent genes: two peptide families with differing disulfide topology share a common ancestry. *Genomics* **43** (1997), pp. 316-20.

Liu, Y., Qin, X., Song, X.Z., Jiang, H., Shen, Y., Durbin, K.J., Lien, S., Kent, M.P., Sodeland, M., Ren, Y., Zhang, L., Sodergren, E., Havlak, P., Worley, K.C., Weinstock, G.M. and Gibbs, R.A. Bos taurus genome assembly. *BMC Genomics* **10** (2009), p. 180.

Lopez-Meza, J.E., Gutierrez-Barroso, A. and Ochoa-Zarzosa, A. Expression of tracheal antimicrobial peptide in bovine mammary epithelial cells. *Research in Veterinary Science* **87** (2009), pp. 59-63.

Lupski, J.R. Charcot-Marie-Tooth disease: lessons in genetic mechanisms. *Mol Med* **4** (1998), pp. 3-11.

Lupski, J.R. and Stankiewicz, P. Genomic disorders: molecular mechanisms for rearrangements and conveyed phenotypes. *PLoS Genet* **1** (2005), p. e49.

Luster, A.D. The role of chemokines in linking innate and adaptive immunity. *Current Opinion in Immunology* **14** (2002), pp. 129-135.

Lynn, D.J. and Bradley, D.G. Discovery of alpha-defensins in basal mammals. *Dev Comp Immunol* **31** (2007), pp. 963-7.

Mackenzie-Dyck, S., Kovacs-Nolan, J., Snider, M., Babiuk, L.A. and van Drunen Littel-van den Hurk, S. Inclusion of the Bovine Neutrophil Beta-Defensin 3 with Glycoprotein D of Bovine Herpesvirus 1 in a DNA Vaccine Modulates Immune Responses of Mice and Cattle. *Clinical and Vaccine Immunology : CVI* **21** (2014), pp. 463-477.

Maestre, J., Tchenio, T., Dhellin, O. and Heidmann, T. mRNA retroposition in human cells: processed pseudogene formation. *EMBO J* **14** (1995), pp. 6333-8.

Malissen, B., Tamoutounour, S. and Henri, S. The origins and functions of dendritic cells and macrophages in the skin. *Nat Rev Immunol* **14** (2014), pp. 417-28.

Mallard, B.A., Emam, M., Paibomesai, M., Thompson-Crispi, K. and Wagter-Lesperance, L. Genetic selection of cattle for improved immunity and health. *Jpn J Vet Res* **63 Suppl 1** (2014), pp. S37-44.

Malmuthuge, N., Li, M., Fries, P., Griebel, P.J. and Guan, L.L. Regional and age dependent changes in gene expression of Toll-like receptors and key antimicrobial defence molecules throughout the gastrointestinal tract of dairy calves. *Vet Immunol Immunopathol* **146** (2012), pp. 18-26.

Mantovani, A., Cassatella, M.A., Costantini, C. and Jaillon, S.b. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* **11** (2011), pp. 519-531.

Maston, G.A., Evans, S.K. and Green, M.R. Transcriptional Regulatory Elements in the Human Genome. *Annual Review of Genomics and Human Genetics* **7** (2006), pp. 29-59.

McCarroll, S.A., Kuruvilla, F.G., Korn, J.M., Cawley, S., Nemesh, J., Wysoker, A., Shapero, M.H., de Bakker, P.I., Maller, J.B., Kirby, A., Elliott, A.L., Parkin, M., Hubbell, E., Webster, T., Mei, R., Veitch, J., Collins, P.J., Handsaker, R., Lincoln, S., Nizzari, M., Blume, J., Jones, K.W., Rava, R., Daly, M.J., Gabriel, S.B. and Altshuler, D. Integrated detection and population-genetic analysis of SNPs and copy number variation. *Nat Genet* **40** (2008), pp. 1166-74.

McManus, C.J. and Graveley, B.R. RNA structure and the mechanisms of alternative splicing. *Current Opinion in Genetics & Development* **21** (2011), pp. 373-379.

Meade, K.G., Cormican, P., Narciandi, F., Lloyd, A. and O'Farrelly, C. Bovine beta-defensin gene family: opportunities to improve animal health? *Physiol Genomics* **46** (2014), pp. 17-28.

Medzhitov, R. and Janeway, C., Jr. Innate immunity. *N Engl J Med* **343** (2000), pp. 338-44.

Mighell, A.J., Smith, N.R., Robinson, P.A. and Markham, A.F. Vertebrate pseudogenes. *FEBS Lett* **468** (2000), pp. 109-14.

Mirabzadeh-Ardakani, A., Griebel, P. and Schmutz, S.M. Identification of a new non-coding exon and haplotype variability in the cattle DEFB103 gene. *Gene* **551** (2014a), pp. 183-8.

Mirabzadeh-Ardakani, A., Griebel, P. and Schmutz, S.M. No association between β-Defensin 103B (DEFB103B) single nucleotide polymorphisms (SNPs) or haplotypes and Staphylococcus aureus mastitis in Holstein cattle. *10th WCGALP* (2014b).

Moore, K.J. Insight into the microphthalmia gene. *Trends Genet* **11** (1995), pp. 442-8.

Mrode, R.A., Swanson, G.J.T. and Winters, M.S. Genetic parameters and evaluations for somatic cell counts and its relationship with production and type traits in some dairy breeds in the United Kingdom. *Animal Science* **66** (1998), pp. 569-576.

Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E. and et, a. Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science* **235** (1987), pp. 1616-1622.

Narciandi, F., Lloyd, A.T., Chapwanya, A., C, O.F. and Meade, K.G. Reproductive tissue-specific expression profiling and genetic variation across a 19 gene bovine beta-defensin cluster. *Immunogenetics* **63** (2011), pp. 641-51.

Nathans, J., Piantanida, T.P., Eddy, R.L., Shows, T.B. and Hogness, D.S. Molecular genetics of inherited variation in human color vision. *Science* **232** (1986), pp. 203-10.

Ng, T.F.F., Kondov, N.O., Deng, X., Van Eenennaam, A., Neibergs, H.L. and Delwart, E. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. *Journal of Virology* (2015).

Nguyen, T.X., Cole, A.M. and Lehrer, R.I. Evolution of primate theta-defensins: a serpentine path to a sweet tooth. *Peptides* **24** (2003), pp. 1647-54.

Nolan, T., Hands, R.E. and Bustin, S.A. Quantification of mRNA using real-time RT-PCR. *Nat Protoc* **1** (2006), pp. 1559-82.

Ohlsen, S.M., Lugenbeel, K.A. and Wong, E.A. Characterization of the linked ovine insulin and insulin-like growth factor-II genes. *DNA Cell Biol* **13** (1994), pp. 377-88.

Ouellette, A.J. and Selsted, M.E. Paneth cell defensins: endogenous peptide components of intestinal host defense. *FASEB J* **10** (1996), pp. 1280-9.

Pan, Q., Shai, O., Lee, L.J., Frey, B.J. and Blencowe, B.J. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat Genet* **40** (2008), pp. 1413-5.

Pancer, Z. and Cooper, M.D. The evolution of adaptive immunity. *Annu Rev Immunol* **24** (2006), pp. 497-518.

Patil, A., Hughes, A.L. and Zhang, G. Rapid evolution and diversification of mammalian alpha-defensins as revealed by comparative analysis of rodent and primate genes. *Physiol Genomics* **20** (2004), pp. 1-11.

Patil, A.A., Cai, Y., Sang, Y., Blecha, F. and Zhang, G. Cross-species analysis of the mammalian beta-defensin gene family: presence of syntenic gene clusters and preferential expression in the male reproductive tract. *Physiol Genomics* **23** (2005), pp. 5-17.

Pazgier, M., Hoover, D.M., Yang, D., Lu, W. and Lubkowski, J. Human beta-defensins. *Cell Mol Life Sci* **63** (2006), pp. 1294-313.

Philipp, U., Lupp, B., Momke, S., Stein, V., Tipold, A., Eule, J.C., Rehage, J. and Distl, O. A MITF mutation associated with a dominant white phenotype and bilateral deafness in German Fleckvieh cattle. *PLoS One* **6** (2011), p. e28857.

Pink, R.C., Wicks, K., Caley, D.P., Punch, E.K., Jacobs, L. and Carter, D.R. Pseudogenes: pseudo-functional or key regulators in health and disease? *RNA* **17** (2011), pp. 792-8.

Piotrowski, A., Bruder, C.E., Andersson, R., Diaz de Stahl, T., Menzel, U., Sandgren, J., Poplawski, A., von Tell, D., Crasto, C., Bogdan, A., Bartoszewski, R., Bebok, Z., Krzyzanowski, M., Jankowski, Z., Partridge, E.C., Komorowski, J. and Dumanski, J.P. Somatic mosaicism for copy number variation in differentiated human tissues. *Hum Mutat* **29** (2008), pp. 1118-24.

Polymeropoulos, M.H., Higgins, J.J., Golbe, L.I., Johnson, W.G., Ide, S.E., Di Iorio, G., Sanges, G., Stenroos, E.S., Pho, L.T., Schaffer, A.A., Lazzarini, A.M., Nussbaum, R.L. and Duvoisin, R.C. Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. *Science* **274** (1996), pp. 1197-9.

Raggo, C., Habermehl, M., Babiuk, L.A. and Griebel, P. The in vivo effects of recombinant bovine herpesvirus-1 expressing bovine interferon-gamma. *J Gen Virol* **81** (2000), pp. 2665-73.

Ramel, C. Mini- and Microsatellites. *Environmental Health Perspectives* **105** (1997), pp. 781-789.

Reddy, K.V., Yedery, R.D. and Aranha, C. Antimicrobial peptides: premises and promises. *Int J Antimicrob Agents* **24** (2004), pp. 536-47.

Redon, R., Ishikawa, S., Fitch, K.R., Feuk, L., Perry, G.H., Andrews, T.D., Fiegler, H., Shapero, M.H., Carson, A.R., Chen, W., Cho, E.K., Dallaire, S., Freeman, J.L., Gonzalez, J.R., Gratacos, M., Huang, J., Kalaitzopoulos, D., Komura, D., MacDonald, J.R., Marshall, C.R., Mei, R., Montgomery, L., Nishimura, K., Okamura, K., Shen, F., Somerville, M.J., Tchinda, J., Valsesia, A., Woodwark, C., Yang, F., Zhang, J., Zerjal, T., Armengol, L., Conrad, D.F., Estivill, X., Tyler-Smith, C., Carter, N.P., Aburatani, H., Lee, C., Jones, K.W., Scherer, S.W. and Hurles, M.E. Global variation in copy number in the human genome. *Nature* **444** (2006), pp. 444-54.

Ribeiro, M.r.G., Salerno, T., Mattos-Guaraldi, A.L.d., Camello, T.C.F., Langoni, H.l., Siqueira, A.K., Paes, A.C., Fernandes, M.C. and Lara, G.H.B. Nocardiosis: an overview and additional report of 28 cases in cattle and dogs. *Revista do Instituto de Medicina Tropical de SÃ£o Paulo* **50** (2008), pp. 177-185.

Rieg, S., Meier, B., Fähnrich, E., Huth, A., Wagner, D., Kern, W.V. and Kalbacher, H. Differential activity of innate defense antimicrobial peptides against Nocardia species. *BMC Microbiology* **10** (2010), pp. 1-8.

Robbins, J.: An Evaluation of the Prostaph I Rapid Diagnostic Test in Detecting Staphylococcus Aureus Mastitis in Holstein Herds, *Animal and Poultry Science*. University of Saskatchewab, saskatoon (1992), pp. 99.

Rouchka, E.C. and Cha, I.E. Current Trends in Pseudogene Detection and Characterization *Current Bioinformatics* **4** (2009), pp. 112-119.

Royster, E. and Wagner, S. Treatment of Mastitis in Cattle. *Vet Clin North Am Food Anim Pract* **31** (2015), pp. 17-46.

Ryan, L.K., Rhodes, J., Bhat, M. and Diamond, G. Expression of Beta-Defensin Genes in Bovine Alveolar Macrophages. *Infection and Immunity* **66** (1998), pp. 878-881.

Ryniewicz, Z., Zwierzchowski, L., Bagnicka, E., Flisikowski, K., Maj, A., Krzyzewski, J. and Strzalkowska, N. Association of the polymorphism at defensin gene loci with dairy production traits and milk somatic cell count in Black-and-White cows. *Anim. Sci. Pap. and Rep* **21** (2003), pp. 209-222.

Sallusto, F. and Lanzavecchia, A. The instructive role of dendritic cells on T-cell responses. *Arthritis Res* **4 Suppl 3** (2002), pp. S127-32.

Sander, P., Hamann, H., Pfeiffer, I., Wemheuer, W., Brenig, B., Groschup, M.H., Ziegler, U., Distl, O. and Leeb, T. Analysis of sequence variability of the bovine prion protein gene (PRNP) in German cattle breeds. *Neurogenetics* **5** (2004), pp. 19-25.

Santoro, D., Bunick, D., Graves, T.K. and Segre, M. Evaluation of canine antimicrobial peptides in infected and noninfected chronic atopic skin. *Veterinary Dermatology* **24** (2013), pp. 39-e10.

Schaschl, H., Aitman, T.J. and Vyse, T.J. Copy number variation in the human genome and its implication in autoimmunity. *Clin Exp Immunol* **156** (2009), pp. 12-6.

Schittek, B., Hipfel, R., Sauer, B., Bauer, J., Kalbacher, H., Stevanovic, S., Schirle, M., Schroeder, K., Blin, N., Meier, F., Rassner, G. and Garbe, C. Dermcidin: a novel human antibiotic peptide secreted by sweat glands. *Nat Immunol* **2** (2001), pp. 1133-7.

Schmutz, S.M., Berryere, T.G. and Dreger, D.L. MITF and White Spotting in Dogs: A Population Study. *Journal of Heredity* **100** (2009), pp. S66-S74.

Seillet, C., Laffont, S., Tremollieres, F., Rouquie, N., Ribot, C., Arnal, J.F., Douin-Echinard, V., Gourdy, P. and Guery, J.C. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol in vivo through cell-intrinsic estrogen receptor alpha signaling. *Blood* **119** (2012), pp. 454-64.

Selsted, M.E. Theta-defensins: cyclic antimicrobial peptides produced by binary ligation of truncated alpha-defensins. *Curr Protein Pept Sci* **5** (2004), pp. 365-71.

Selsted, M.E., Harwig, S.S., Ganz, T., Schilling, J.W. and Lehrer, R.I. Primary structures of three human neutrophil defensins. *J Clin Invest* **76** (1985), pp. 1436-9.

Selsted, M.E., Tang, Y.Q., Morris, W.L., McGuire, P.A., Novotny, M.J., Smith, W., Henschen, A.H. and Cullor, J.S. Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils. *Journal of Biological Chemistry* **268** (1993), pp. 6641-6648.

Shimoda, M., Ohki, K., Shimamoto, Y. and Kohashi, O. Morphology of defensin-treated Staphylococcus aureus. *Infect Immun* **63** (1995), pp. 2886-91.

Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M.R., Muenter, M., Baptista, M., Miller, D.,

Blancato, J., Hardy, J. and Gwinn-Hardy, K. alpha-Synuclein locus triplication causes Parkinson's disease. *Science* **302** (2003), p. 841.

Singleton, A.B., Farrer, M.J. and Bonifati, V. The genetics of Parkinson's disease: progress and therapeutic implications. *Mov Disord* **28** (2013), pp. 14-23.

Smale, S.T. and Kadonaga, J.T. The RNA polymerase II core promoter. *Annu Rev Biochem* **72** (2003), pp. 449-79.

Smith, S.D., Kelley, P.M., Kenyon, J.B. and Hoover, D. Tietz syndrome (hypopigmentation/deafness) caused by mutation of MITF. *J Med Genet* **37** (2000), pp. 446-8.

Sorensen, O.E., Cowland, J.B., Theilgaard-Monch, K., Liu, L., Ganz, T. and Borregaard, N. Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J Immunol* **170** (2003), pp. 5583-9.

Stankiewicz, P. and Lupski, J.R. Structural variation in the human genome and its role in disease. *Annu Rev Med* **61** (2010), pp. 437-55.

Steingrimsson, E., Copeland, N.G. and Jenkins, N.A. Melanocytes and the microphthalmia transcription factor network. *Annu Rev Genet* **38** (2004), pp. 365-411.

Steingrimsson, E., Moore, K.J., Lamoreux, M.L., Ferre-D'Amare, A.R., Burley, S.K., Zimring, D.C., Skow, L.C., Hodgkinson, C.A., Arnheiter, H., Copeland, N.G. and et al. Molecular basis of mouse microphthalmia (mi) mutations helps explain their developmental and phenotypic consequences. *Nat Genet* **8** (1994), pp. 256-63.

Stewart, R.J., Hill, F.W., Masztalerz, A., Jacobs, J.J., Koten, J.W. and Den Otter, W. Treatment of ocular squamous cell carcinomas in cattle with interleukin-2. *Vet Rec* **159** (2006), pp. 668-72.

Strom, S.A.: Characterization and mapping of Microphtalmia-associated transcription factor (MITF) in cattle., *Animal and Pultry Science*. University of Saskatchewan, Saskatoon (2003), pp. 103.

Suzuki, Y., Tsunoda, T., Sese, J., Taira, H., Mizushima-Sugano, J., Hata, H., Ota, T., Isogai, T., Tanaka, T., Nakamura, Y., Suyama, A., Sakaki, Y., Morishita, S., Okubo, K. and Sugano, S. Identification and characterization of the potential promoter regions of 1031 kinds of human genes. *Genome Res* **11** (2001), pp. 677-84.

Swanson, K., Gorodetsky, S., Good, L., Davis, S., Musgrave, D., Stelwagen, K., Farr, V. and Molenaar, A. Expression of a B-Defensin mRNA, Lingual Antimicrobial Peptide, in Bovine Mammary Epithelial Tissue Is Induced by Mastitis. *Infection and Immunity* **72** (2004), pp. 7311-7314.

Tang, Y.Q. and Selsted, M.E. Characterization of the disulfide motif in BNBD-12, an antimicrobial beta-defensin peptide from bovine neutrophils. *J Biol Chem* **268** (1993), pp. 6649-53.

Tang, Y.Q., Yuan, J., Osapay, G., Osapay, K., Tran, D., Miller, C.J., Ouellette, A.J. and Selsted, M.E. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* **286** (1999), pp. 498-502.

Tassabehji, M., Newton, V.E. and Read, A.P. Waardenburg syndrome type 2 caused by mutations in the human microphthalmia (MITF) gene. *Nat Genet* **8** (1994), pp. 251-5.

Thomma, B.P., Cammue, B.P. and Thevissen, K. Plant defensins. *Planta* **216** (2002), pp. 193-202.

Thompson-Crispi, K., Atalla, H., Miglior, F. and Mallard, B.A. Bovine Mastitis: Frontiers in Immunogenetics. *Frontiers in Immunology* **5** (2014a), p. 493.

Thompson-Crispi, K., Atalla, H., Miglior, F. and Mallard, B.A. Bovine mastitis: frontiers in immunogenetics. *Front Immunol* **5** (2014b), p. 493.

Thompson-Crispi, K.A., Hine, B., Quinton, M., Miglior, F. and Mallard, B.A. Short communication: Association of disease incidence and adaptive immune response in Holstein dairy cows. *J Dairy Sci* **95** (2012), pp. 3888-93.

Thompson-Crispi, K.A., Sargolzaei, M., Ventura, R., Abo-Ismail, M., Miglior, F., Schenkel, F. and Mallard, B.A. A genome-wide association study of immune response traits in Canadian Holstein cattle. *BMC Genomics* **15** (2014c), p. 559.

Trowsdale, J. and Knight, J.C. Major histocompatibility complex genomics and human disease. *Annu Rev Genomics Hum Genet* **14** (2013), pp. 301-23.

Tsai, H. and Bobek, L.A. Human salivary histatins: promising anti-fungal therapeutic agents. *Crit Rev Oral Biol Med* **9** (1998), pp. 480-97.

Tsujita, H. and Plummer, C.E. Bovine ocular squamous cell carcinoma. *Vet Clin North Am Food Anim Pract* **26** (2010), pp. 511-29.

Udono, T., Yasumoto, K., Takeda, K., Amae, S., Watanabe, K., Saito, H., Fuse, N., Tachibana, M., Takahashi, K., Tamai, M. and Shibahara, S. Structural organization of the human microphthalmia-associated transcription factor gene containing four alternative promoters. *Biochim Biophys Acta* **1491** (2000), pp. 205-19.

Valero, M., Richerd, S., Perrot, V.r. and Destombe, C. Evolution of alternation of haploid and diploid phases in life cycles. *Trends in Ecology & Evolution* **7** (1992), pp. 25-29.

Vanin, E.F. Processed Pseudogenes: Characteristics and Evolution. *Annual Review of Genetics* **19** (1985), pp. 253-272.

Vincent, J.B., Herbrick, J.A., Gurling, H.M., Bolton, P.F., Roberts, W. and Scherer, S.W. Identification of a novel gene on chromosome 7q31 that is interrupted by a translocation breakpoint in an autistic individual. *Am J Hum Genet* **67** (2000), pp. 510-4.

Wagter, L.C. and Mallard, B.A.: Method of identifying high immune response animals. University of Guelph, Guelph, ON, Canada, assignee. US Pat. No. 7,258,858. (2007).

Wagter, L.C., Mallard, B.A., Wilkie, B.N., Leslie, K.E., Boettcher, P.J. and Dekkers, J.C. A quantitative approach to classifying Holstein cows based on antibody responsiveness and its relationship to peripartum mastitis occurrence. *J Dairy Sci* **83** (2000), pp. 488-98.

Wang, E.T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., Kingsmore, S.F., Schroth, G.P. and Burge, C.B. Alternative isoform regulation in human tissue transcriptomes. *Nature* **456** (2008), pp. 470-6.

Wang, X., Xu, S., Gao, X., Ren, H. and Chen, J. Genetic Polymorphism of TLR4 Gene and Correlation with Mastitis in Cattle. *Journal of Genetics and Genomics* **34** (2007), pp. 406-412.

Whale, T.A., Beskorwayne, T.K., Babiuk, L.A. and Griebel, P.J. Bovine polymorphonuclear cells passively acquire membrane lipids and integral membrane proteins from apoptotic and necrotic cells. *J Leukoc Biol* **79** (2006), pp. 1226-33.

Whelehan, C.J., Meade, K.G., Eckersall, P.D., Young, F.J. and Oâ€™Farrelly, C. Experimental Staphylococcus aureus infection of the mammary gland induces region-specific changes in innate immune gene expression. *Veterinary Immunology and Immunopathology* **140** (2011), pp. 181-189.

Widlund, H.R. and Fisher, D.E. Microphthalamia-associated transcription factor: a critical regulator of pigment cell development and survival. *Oncogene* **22** (2003), pp. 3035-41.

Woodwark, C. and Bateman, A. The characterisation of three types of genes that overlie copy number variable regions. *PLoS One* **6** (2011), p. e14814.

Xin, D., Hu, L. and Kong, X. Alternative promoters influence alternative splicing at the genomic level. *PLoS One* **3** (2008), p. e2377.

Yang, D., Biragyn, A., Kwak, L.W. and Oppenheim, J.J. Mammalian defensins in immunity: more than just microbicidal. *Trends in Immunology* **23** (2002), pp. 291-296.

Yang, D., Chertov, O., Bykovskaia, S.N., Chen, Q., Buffo, M.J., Shogan, J., Anderson, M., Schroder, J.M., Wang, J.M., Howard, O.M.Z. and Oppenheim, J.J. B-Defensins: Linking Innate and Adaptive Immunity Through Dendritic and T Cell CCR6. *Science* **286** (1999), pp. 525-528.

Yasumoto, K., Amae, S., Udono, T., Fuse, N., Takeda, K. and Shibahara, S. A big gene linked to small eyes encodes multiple Mitf isoforms: many promoters make light work. *Pigment Cell Res* **11** (1998), pp. 329-36.

Yee, J.Y., Limenta, L.M.G., Rogers, K., Rogers, S.M., Tay, V.S.Y. and Lee, E.J.D. Ensuring good quality rna for quantitative real-time pcr isolated from renal proximal tubular cells using laser capture microdissection. *BMC Research Notes* **7** (2014), pp. 62-62.

Zak, D.E. and Aderem, A. Systems integration of innate and adaptive immunity. *Vaccine* **33** (2015), pp. 5241-5248.

Zanetti, M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* **75** (2004), pp. 39-48.

Zanetti, M., Gennaro, R. and Romeo, D. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Lett* **374** (1995), pp. 1-5.

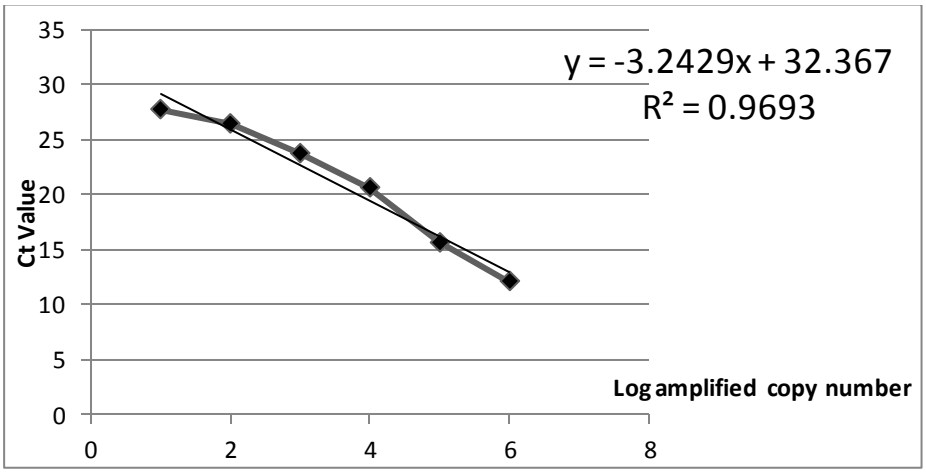
Zhang, F., Gu, W., Hurles, M.E. and Lupski, J.R. Copy Number Variation in Human Health, Disease, and Evolution. *Annual Review of Genomics and Human Genetics* **10** (2009), pp. 451-481.

Zimin, A.V., Delcher, A.L., Florea, L., Kelley, D.R., Schatz, M.C., Puiu, D., Hanrahan, F., Pertea, G., Van Tassell, C.P., Sonstegard, T.S., Marcais, G., Roberts, M., Subramanian, P., Yorke, J.A. and Salzberg, S.L. A whole-genome assembly of the domestic cow, Bos taurus. *Genome Biol* **10** (2009), p. R42.

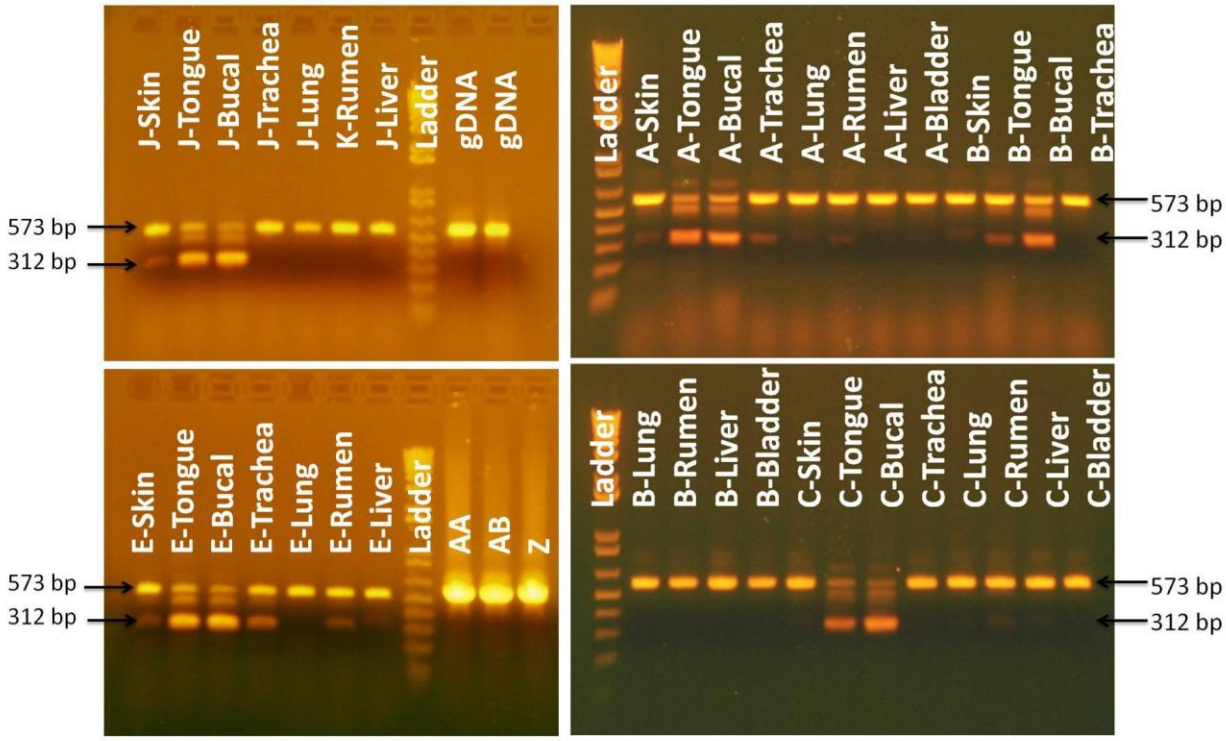
Zimin, A.V., Kelley, D.R., Roberts, M., Marcais, G., Salzberg, S.L. and Yorke, J.A. Mis-assembled "segmental duplications" in two versions of the Bos taurus genome. *PLoS One* **7** (2012), p. e42680.

8.0 APPENDICES

Appendix A: Standard curve for primer validation



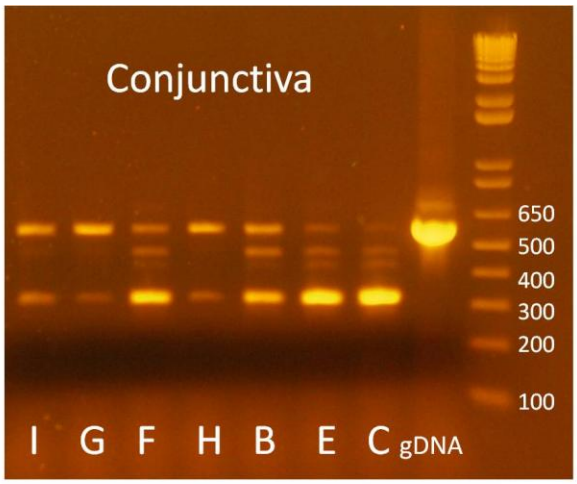
Appendix B: Photographs of 1% agarose gels showing a 573 bp partial cDNA (including intron 1a) and a 312 bp partial cDNA (intron 1a spliced out) when KNEST1-F and DEFEX1-R primers were used from 6 cattle (A, B, C, E, J, K).



Primers	KNEST1-F and DEFEX1-R
Genomic product	573 bp
cDNA product	312 bp

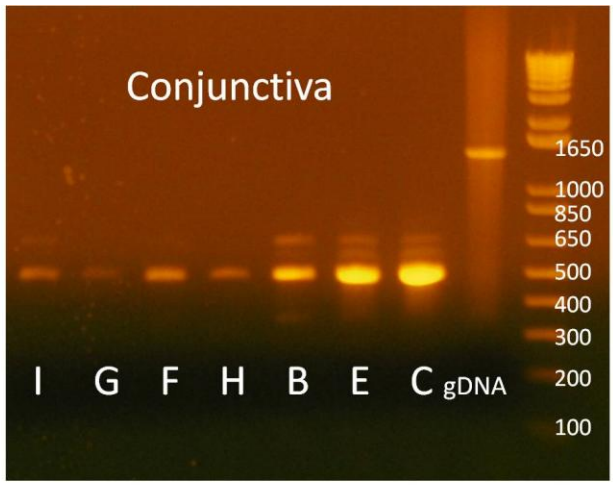
Appendix C: Photographs of 1% agarose gels showing a 511 bp complete cDNA transcript when (a) KNEST1-F and DEFEX1-R and (b) KNEST1-F and DEFB103EX2-R2 primers were used (illustrating potential alternative splicing in seven different cattle).

a



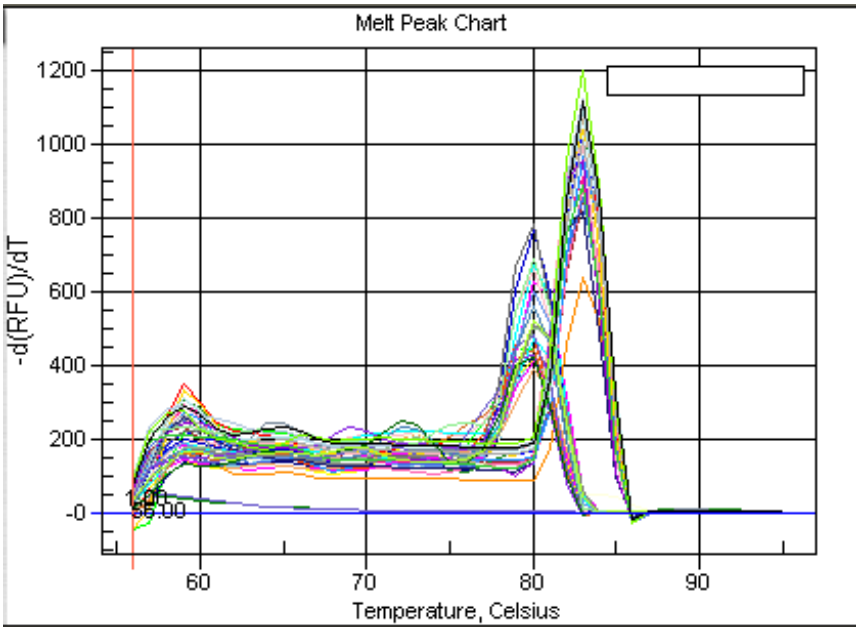
Primers	KNEST1-F and DEFEX1-R
Genomic product	573 bp
cDNA product	312 bp

b



Primers	KNEST1-F and DEFB103EX2-R2
Genomic product	1432 bp
cDNA product	511 bp

Appendix D: Melting Curve for *DEFB103* and *B-actin* products



Appendix E: Abstract for the Annual Life & Health Sciences Research Day March 14th, 2014, University of Saskatchewan, Saskatoon

No association between *β-Defensin 103B (DEFB103B)* mutations or haplotype and Cancer-Eye in Hereford cattle

A. Mirabzadeh-Ardakani[1], P. Griebel[2], and S. M. Schmutz[1]

Ocular squamous cell carcinoma or "Cancer-eye" commonly occurs in the epithelial surfaces of conjunctiva. Cancer-Eye is more common in cattle breeds with unpigmented skin around the eyes, such as Hereford. It typically has late onset, with most affected cattle being greater than 7-9 years of age. It is a rapidly developing cancer that often results in the necessity of humane euthanasia within months. One of the important components of innate host defenses is antimicrobial peptide production, with cattle *β-Defensin 103B* (*DEFB103B*) being one of these peptides. Cattle *DEFB103B* in cattle is homologous to human *DEFB3*. In this study, Cancer-eye was identified by an inspecting veterinarian at a slaughter facility. A tissue survey of *DEFB103B* gene expression revealed epithelial tissues, including the conjunctiva, have the highest level of *DEFB103B* gene expression. Six single nucleotide polymorphisms (SNP) were identified in the 5'UTR of *DEFB103B* in beef cattle, and four of these are present in cDNA. These SNPs occurred in two haplotypes in the 49 Cancer-eye DNA samples from Hereford cattle. We hypothesized that a specific *DEFB103B* haplotype may be associated with the relative risk of Cancer-eye. Haplotype and allelic frequencies were analyzed for cattle affected with Cancer-eye and healthy controls. All possible combinations of *DEFB103B* haplotypes were identified among the samples. There was no significant difference in allelic or haplotype frequencies among the 49 Hereford cattle with cancer-eye when compared to 40 healthy beef cattle (10 animals each from four major beef cattle breeds: Angus, Hereford, Simmental, Charolais). The minor allele frequency for SNPs in Cancer-eye samples was 26%, and in healthy beef cattle was 33.75%. Although the 40 "control" samples are from cattle of various ages, we conclude that sampling age-matched healthy Hereford cattle samples as a control group, would not alter the *DEFB103B* allele frequency in animals free of Cancer-eye.

Appendix F: Poster presented in the Annual Life & Health Sciences Research Day March 14th, 2014, University of Saskatchewan, Saskatoon

No association between *β-Defensin 103B (DEFB103B)* mutations or haplotypes and Cancer-Eye in Hereford cattle

A. Mirabzadeh-Ardakani[1], P. Griebel[2], and S. M. Schmutz[1]
[1]Department of Animal and Poultry Science, University of Saskatchewan, Canada,
[2]Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Canada

Introduction

Ocular squamous cell carcinoma or "Cancer-eye" commonly occurs in the epithelial surfaces of the conjunctiva (Figure 1). Cancer-Eye is more common in cattle breeds with unpigmented skin around the eyes, such as Hereford (Stewart *et al.*, 2006).

It typically has late onset, with most affected cattle being greater than 7-9 years of age (Tsujita and Plummer, 2010).

One of the important components of innate host defenses is antimicrobial peptide production, like the *DEFB103B*. Cattle *DEFB103B* is homologous to human *DEFB3*.

Single nucleotide polymorphisms have been reported in the 5′ UTR of this gene (Dereger and Schmutz, 2009) (Figure 2).

A tissue survey of *DEFB103B* gene expression revealed that epithelial tissues, including the conjunctiva, have the highest levels of *DEFB103B* gene expression (unpublished data).

Objective

Test for an association of *DEFB103B* single nucleotide polymorphisms or haplotypes and Cancer-eye in Hereford cattle.

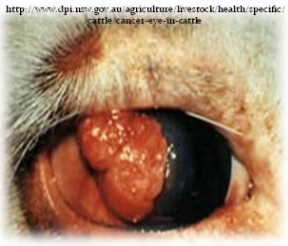


Figure 1: Photograph of an eye of a Hereford cow affected with Ocular squamous cell carcinoma (Cancer-eye)

Methods

- The *DEFB103B* gene occurs in multiple copies in cattle (Elsik *et al.*, 2009), so a semi-nested PCR was used to exclude multiple products or pseudogenes.
- In the primary PCR reaction KNEST1-F and KNEST1-R primers were used.
- The primary PCR product was used as a template for the semi-nested PCR.
- In the semi-nested amplification reaction the same forward primer (KNEST1-F) and KDEFEX1-R primer were used.
- Sequencing was used to determine the genotype of each cow for the 7 SNPs in the 5′ UTR of beef cattle *DEFB103B*.
- Haplotype and allelic frequencies were analyzed for those cattle affected with Cancer-eye and healthy controls by using a Fisher Exact probability test.

Results

- These 7 SNPs occurred in two haplotypes (2 and 4) in Hereford cattle.
- All possible combinations of these *DEFB103B* haplotypes were identified among these cows (2/2, 2/4, and 4/4) (Table 1).
- No significant difference (P = 0.43) was found between the frequency of haplotypes in Hereford cattle with Cancer-eye and Healthy beef cattle.

Table 1: Haplotype frequencies in Hereford cattle with Cancer-eye and healthy beef cattle (10 animals from four major beef cattle breeds: Angus, Hereford, Simmental, Charolais)

Haplotype	Hereford cattle with Cancer-eye		Healthy beef cattle	
	n	Frequency	n	Frequency
2/2	27	0.55	19	0.47
2/4	19	0.39	15	0.38
4/4	3	0.06	6	0.15
Total	49		40	

Conclusion

- Since the *DEFB103B* gene occurs in multiple copies in cattle (Elsik *et al.*, 2009), different copy number between the cows can make them more susceptible or more resistant to the cancer-eye.
- DEFB103 gene expression was high in the conjunctiva, which suggested that this gene might play a role in immune function there.
- **However, the SNP haplotypes differences were not associated with resistance or susceptibility to Cancer-Eye.**

Haplotype	-383	-319	-264	-241	-69	-42	-34	Presence
1	A	G	T	G	G	G	A	Holsteins only
2	**A**	**G**	**T**	A	**G**	**G**	**A**	Holstein and beef cattle
3	A	A	C	A	A	A	G	Holsteins only
4	**G**	**A**	**C**	A	**A**	**A**	**G**	Holstein and beef cattle

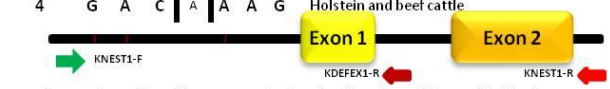


Figure 2: The Position of the *DEFB103B* single nucleotide polymorphisms and their haplotypes.

References
- Dreger, D. L., and S. M. Schmutz. 2009. Anim Genet 41: 109-112.
- Elsik, C. G. *et al.* 2009. Science 324: 522-528.
- Stewart, R. J. *et al.* 2006. Vet Rec 159: 668-672.
- Tsujita, H., and C. E. Plummer. 2010. Vet Clin North Am Food Anim Pract 26: 511-529.

Appendix G: Extended abstract presented in the 10th World Congress of Genetics Applied to Livestock Production (WCGALP), August 17–22, 2014, Vancouver, BC.

Proceedings, 10th World Congress of Genetics Applied to Livestock Production

No association between *β-Defensin 103B (DEFB103B)* single nucleotide polymorphisms (SNPs) or haplotypes and *Staphylococcus aureus* mastitis in Holstein cattle

A. Mirabzadeh-Ardakani*, P. J. Griebel†, and S. M. Schmutz*.
*Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Canada,
†Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Canada

ABSTRACT: *Staphylococcus aureus* infection was previously monitored in two populations of Holstein cattle from Saskatchewan (Canada) and Pennsylvania (USA). All cows were genotyped for seven SNPs present in the 5'UTR of cattle *DEFB103B*. Cows with the diploid haplotype 1/4 (P = 0.053) tended toward an increased risk of mastitis in the Saskatchewan population. Cows with the diploid haplotype 4/4 (P = 0.06) tended toward a decreased risk of mastitis in the Pennsylvania population. Relative risk and odds ratio results indicated that haploid haplotypes 2 and 3 in the Saskatchewan population, and haploid haplotypes 1 and 4 in the Pennsylvania population were less susceptible to mastitis. However, because the results of the Fisher Exact test, the Relative risk and the Odds ratio were not consistent between the two populations, these *DEFB103B* haplotypes cannot be used as markers to select for mastitis resistance.
Keywords: Beta Defensin gene family; CNV; dairy cattle

Introduction

The *β-Defensin* gene family includes a gene cluster on chromosome 27 in cattle (Gallagher et al. (1995)). These genes encode small cationic peptides with antimicrobial activity (Ganz (2003)). These proteins play an important role in the innate immune system that is the first barrier of defense against pathogen attack. Combined defensin band patterns have been determined by using a PCR-RFLP of different *β-Defensin* genes (Ryniewicz et al. (2003)). These Combined defensin band patterns had a significant association with a low somatic cell count (Ryniewicz et al. (2003)).

The *DEFB103B* is expressed at high levels in the epithelial cells of human (Pazgier et al. (2006)), dog (Leonard et al. (2012)), and cattle (Elsik et al. (2009)). Five SNPs (c.-34A>G, c.-42A>G, c.-69A>G, c.-264C>T, and c.-319A>G) were previously reported in the 5' UTR of cattle *DEFB103B* (Dreger and Schmutz (2009)). The purpose of this study was to evaluate the association of these *DEFB103B* SNPs, plus two additional SNPs discovered in this study, and their haplotypes, with mastitis in two populations of Holstein cattle.

Materials and Methods

Animals and mastitis diagnosis. The Saskatchewan population (n=113) was composed of Holstein cattle from three herds. The Pennsylvania population, consisting of four herds (n=92), was used as the validation population. In previous related studies, these herds were chosen for their high incidence of mastitis, and their management practices that exposed all cows to the risk of infection. Milk samples (3-5 ml) were collected from each quarter of each cow, twice, with one month interval. Cows which were infected with *S. aureus* on both samplings were classified as having mastitis.

Genotyping. The *DEFB103B* gene occurs in multiple copies in cattle (Elsik et al. (2009)), so a semi-nested PCR was used to amplify a specific fragment of the *DEFB103B,* in an attempt to exclude multiple products or pseudogenes. In the primary PCR reaction a 1925 base pair fragment of *DEFB103B* was amplified by forward (5'-GCGTATCCATGCAGGTTCAG-3') and reverse (5'-AGGCCTGAGGTCAGTCAGAACA-3') primers, with an annealing temperature of 63°C. The primary PCR product was used as a template for the semi-nested PCR. In the semi-nested amplification reaction the same forward primer and another reverse primer (5'-GATTAGTCTCTTAAG AGTGAATCAGAA-3') were used with an annealing temperature of 60°C to amplify a 787 base pair fragment. Sequencing was used to determine the genotype of each cow for the seven SNPs in the 5' UTR of cattle *DEFB103B*.

Statistical Analysis. GraphPad Prism 6, version 6.02 was used to analyze the data. A two-tailed Fisher Exact test was used to determine if there was a significant difference in the presence or absence of *S. aureus* among the cattle of each diploid haplotype. To determine if a particular haplotype might be acting as a dominant allele to impart resistance or susceptibility to *S. aureus*, a Fisher Exact test was performed to compare the number of individuals with each haplotype versus those individuals who did not have that haplotype, with or without mastitis. Relative risk (RR) and odds ratios (OR) (95% confidence interval) were calculated. Relative risk and/or odds ratios are often reported for a particular genotype or haplotype that may impart some level of resistance to a disease (Lang and Secic (2006)). $P<0.05$ was considered statistically significant.

Results and Discussion

Two new SNPs (c.-241G>A, and c.-383A>G) were identified in this study (Genbank KF545952.1). Four haplotypes were determined based on the five SNPs that had been reported by Dreger and Schmutz (2009) and these two new SNPs, and are presented in Table 1.

Table 1. The four haplotypes were determined based on the seven SNPs at the 5'UTR of *DEFB103B* in the Holstein cattle.

Haplotype	SNPs						
	-383	-319	-264	-241	-69	-42	-34
1	A	G	T	G	G	G	A
2	A	G	T	A	G	G	A
3	A	A	C	A	A	A	G
4	G	A	C	A	A	A	G

There was a trend toward increased risk of mastitis in cows with the 1/4 diploid haplotype (P = 0.053), in the Saskatchewan population, when compared to individuals with other haplotypes (Table 2). In the Pennsylvania population, there was a trend toward decreased risk of mastitis in cows with the 4/4 diploid haplotype (P = 0.06), when compared to other haplotypes (Table 2). These trends were not validated by both populations.

In the Saskatchewan population, the number of individuals that had at least one copy of haplotype 2 (P = 0.09) showed a trend toward decreased risk of mastitis. On the other hand, the number of individuals that had at least one copy of haplotype 4 (P = 0.09), showed a trend toward increased risk of mastitis (Table 3). The relative risk and the odds ratios results (Table 3) indicate that, individuals with *S. aureus* infection in the Saskatchewan population, with haploid haplotypes 2 and 3, and in the Pennsylvania population with haploid haplotypes 1 and 4 were less susceptible to mastitis. There was no consistent diploid or haploid haplotype that imparted resistance or susceptibility to *S. aureus* between the two populations.

DEFB103B has multiple copies in human (Hollox et al. (2003)), dog (Leonard et al. (2012)), and cattle (Elsik et al. (2009)). Fode et al. (2011) reported that *S. aureus* carrier status in humans is not affected by the *DEFB103B* copy number variation (CNV). All of these results may suggest that the contribution of the *DEFB103B* haplotypes to disease resistance or susceptibility varies with the impact of other environmental factors which contribute greatly to the risk of *S. aureus* infection (Keefe (2012)).

Table 2. The *DEFB103B* diploid haplotypes in cattle with *S. aureus* mastitis. Two-tailed P-values are reported based on a Fisher Exact test.

Haplotype	Saskatchewan Population			Pennsylvania Population		
	Mastitis	No Mastitis	P	Mastitis	No Mastitis	P
1/1	1	1	0.49	0	0	-
1/2	3	8	0.99	1	1	0.37
1/3	1	4	0.99	0	2	0.99
1/4	4	2	0.05	0	3	0.99
2/2	2	12	0.34	4	7	0.23
2/3	0	7	0.19	3	8	0.69
2/4	7	19	0.99	6	24	0.99
3/3	1	3	0.99	0	3	0.99
3/4	7	14	0.60	5	12	0.33
4/4	6	11	0.56	0	13	0.06
Total	32	81		19	73	

Table 3. The *DEFB103B* haploid haplotypes of cattle with and without *S. aureus* mastitis. Two-tailed P-values are reported based on a Fisher Exact test. Yes means the presence of haplotype and No means the absence of haplotype.

Haplotype	P	Mastitis		No Mastitis		Relative Risk (95% CI)	Odds Ratio (95% CI)
		Yes	No	Yes	No		
Saskatchewan Population							
1	0.31	9	23	15	66	1.52 (0.74-3.11)	1.72 (0.66-4.47)
2	0.09	12	20	46	35	0.66 (0.41-1.07)	0.46 (0.20-1.06)
3	0.66	9	23	28	53	0.81 (0.43-1.53)	0.74 (0.30-1.82)
4	0.09	24	8	46	35	1.32 (1.00-1.74)	2.28 (0.92-5.69)
Pennsylvania Population							
1	0.99	1	18	6	67	0.64 (0.08-5.00)	0.62 (0.07-5.49)
2	0.19	14	5	40	33	1.34 (0.96-1.89)	2.31 (0.75-7.08)
3	0.59	8	11	25	48	1.30 (0.66-2.28)	1.40 (0.50-3.92)

Acknowledgments

This study was funded by the Saskatchewan Agriculture Development Fund. We thank Jim Robbins and Lorraine Sordillo who collected milk samples and mastitis data during a previous study.

Literature Cited

Dreger, D.L., and Schmutz, S.M. (2009). Anim. Genet., 41:109-12.
Elsik, C.G., Tellam, R.L., Worley, K.C. et al. (2009). Science, 324:522-528
Fode, P., Stegger, M., and Andersen, P.S. (2011). Int. J. Infect. Dis., 15:388-94
Gallagher, DS.Jr., Ryan, AM., Diamond, G. et al. (1995). Mamm. Genome, 6:554-6.
Ganz, T. (2003). Nat. Rev. Immunol., 3:710-20.
Hollox, E.J., Armour, J.A.L., and Barber, J.C.K. (2003). Am. J. Hum. Genet., 73:591–600
Keefe, G. (2012). Vet. Clin. North Am. Food Anim. Pract., 28:203-16.
Lang, T.A. & Secic M. (2006). 2006, 490, American College of Physicians, Philadelphia, 2.
Leonard, B.C., Marks, S.L., Outerbridge, C.A. et al. (2012). J. Innate. Immun., 4:248–259
Pazgier, M., Hoover, DM., Yang, D. et al. (2006). Cell. Mol. Life Sci., 63:1294-313.
Ryniewicz, Z., Zwierzchowski, L., Bagnicka, E. et al. (2003). Anim. Sci. Papers and Reports, 21:209-222.

Appendix H: Poster presented in the 10th World Congress of Genetics Applied to Livestock Production (WCGALP), August 17–22, 2014, Vancouver, BC.

No association between *β-Defensin 103B (DEFB103B)* single nucleotide polymorphisms (SNPs) or haplotypes and *Staphylococcus aureus* mastitis in Holstein cattle

#528

A. Mirabzadeh-Ardakani[1], P. J. Griebel[2], and S. M. Schmutz[1]

[1]Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Canada

[2]Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Canada

Introduction

- The *β-Defensin* gene family includes a gene cluster on chromosome 27 in cattle (Gallagher *et al.*, 1995).
- Combined defensin band patterns have been determined by using a PCR-RFLP of different *β-Defensin* genes, and a significant association with a low somatic cell count was found (Ryniewicz *et al.*, 2003).
- Five SNPs (c.-34A>G, c.-42A>G, c.-69A>G, c.-264C>T, and c.-319A>G) were previously reported in the 5′UTR of cattle *DEFB103B* (Dreger and Schmutz, 2009).
- *DEFB103B* is expressed at high levels in the epithelial cells of humans (Pazgier *et al.*, 2006), dogs (Leonard *et al.*, 2012), and cattle (unpublished data).

Objective

The purpose of this study was to evaluate the association of *DEFB103B* haplotypes with mastitis in two populations of Holstein cattle.

Methods

- A Saskatchewan population (n=113, 3 herds), and a Pennsylvania population, (n=92, 4 herds) were genotyped for *DEFB103B* SNPs in the 5′UTR, by sequencing.
- *DEFB103B* gene occurs in multiple copies in cattle (Elsik *et al.*, 2009), so a semi-nested PCR (Figure 1) was used in an attempt to exclude multiple products or pseudogenes.
- A two-tailed Fisher Exact test was used to analyze the data. P<0.05 was considered statistically significant.
- Relative risk and odds ratios were calculated (Lang and Secic, 2006).

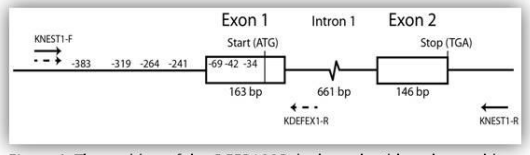


Figure 1: The position of the *DEFB103B* single nucleotide polymorphisms.

Results

- Two new SNPs (c.-241G>A, and c.-383A>G) were identified in this study (Genbank KF545952.1).
- Four haplotypes were determined based on the five SNPs that had been reported by Dreger and Schmutz (2009) and these two new SNPs.

Table 1. The *DEFB103B* **diploid haplotypes** in cattle with *S. aureus* mastitis.

Haplotype	Saskatchewan Population			Pennsylvania Population		
	Mastitis	No Mastitis	P	Mastitis	No Mastitis	P
1/1	1	1	0.49	0	0	-
1/2	3	8	0.99	1	1	0.37
1/3	1	4	0.99	0	2	0.99
1/4	4	2	0.05	0	3	0.99
2/2	2	12	0.34	4	7	0.23
2/3	0	7	0.19	3	8	0.69
2/4	7	19	0.99	6	24	0.99
3/3	1	3	0.99	0	3	0.99
3/4	7	14	0.60	5	12	0.33
4/4	6	11	0.56	0	13	0.06
Total	32	81		19	73	

Table 2. The *DEFB103B* **haploid haplotypes** of cattle with and without *S. aureus* mastitis.

Haplotype	P	Mastitis		No Mastitis		Relative Risk (95% CI)	Odds Ratio (95% CI)
		Yes	No	Yes	No		
Saskatchewan							
1	0.31	9	23	15	66	1.52 (0.74-3.11)	1.72 (0.66-4.47)
2	0.09	12	20	46	35	0.66 (0.41-1.07)	0.46 (0.20-1.06)
3	0.66	9	23	28	53	0.81 (0.43-1.53)	0.74 (0.30-1.82)
4	0.09	24	8	46	35	1.32 (1.00-1.74)	2.28 (0.92-5.69)
Pennsylvania							
1	0.99	1	18	6	67	0.64 (0.08-5.00)	0.62 (0.07-5.49)
2	0.19	14	5	40	33	1.34 (0.96-1.89)	2.31 (0.75-7.08)
3	0.59	8	11	25	48	1.30 (0.66-2.28)	1.40 (0.50-3.92)
4	0.28	11	8	52	21	0.63 (0.54-1.22)	0.56 (0.20-1.57)

Conclusion

- There was no consistent diploid or haploid haplotype that imparted resistance or susceptibility to *S. aureus* in both populations.
- There was inconsistent relative risk of mastitis in both populations.

References

- Dreger, D.L., and Schmutz, S.M. (2009). Anim. Genet., 41:109-12.
- Elsik, C.G., Tellam, R.L., Worley, K.C. et al. (2009). Science, 324:522-528
- Gallagher, DS.Jr., Ryan, A.M., Diamond, G. et al. (1995). Mamm. Genome, 6:554-6.
- Leonard, B.C., Marks, S.L., Outerbridge, C.A. et al. (2012). J. Innate. Immun., 4:248–259.
- Lang, T.A. & Seck M. (2006). 2006. 490. American College of Physicians, Philadelphia, 2.
- Pazgier, M., Hoover, D.M., Yang, D. et al. (2006). Cell. Mol. Life Sci., 63:1294-313.
- Ryniewicz, Z., Zwierzchovski, L., Bagnicka, E. et al. (2003). Anim. Sci. Papers and Reports, 21:209-222.

Appendix I: Manuscript published in *Gene*. Mirabzadeh-Ardakani, A., Griebel, P. and Schmutz, S.M. Identification of a new non-coding exon and haplotype variability in the cattle DEFB103 gene. *Gene* **551** (2014a), pp. 183-8.

Gene 551 (2014) 183–188

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Identification of a new non-coding exon and haplotype variability in the cattle *DEFB103* gene

Ali Mirabzadeh-Ardakani [a,*], Philip Griebel [b], Sheila M. Schmutz [a]

[a] Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Canada
[b] Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, Canada

ARTICLE INFO

Article history:
Received 30 June 2014
Received in revised form 15 August 2014
Accepted 26 August 2014
Available online 27 August 2014

Keywords:
Copy number variation
Defensin
Haplotype
Splicing

ABSTRACT

The *DEFB103* gene is a member of the β-defensin gene family. In this study, we applied multiple sets of primers to characterize the *DEFB103* transcript. RT-PCR was used to determine the cDNA boundaries and it indicated that the cDNA start point is at least 514 bp before the start codon and not further than 678 bp. In addition, the length of the 3′UTR was determined to be at least 53 bp after the stop codon. Seven SNPs were located in the 5′UTR, and comprised 4 different haplotypes in genomic DNA. Using these haplotype data, it could be proven that at least two complete copies of *DEFB103* with an ATG start codon are present in cDNA in most cattle. Additionally haplotype data indicated that there are also multiple incomplete copies in most cattle. A non-coding exon 1a, and a 261 bp intron 1a were identified in cattle, and subsequently predicted in sheep and goats. *DEFB103* sequence assemblies and partial cloning sequences revealed two types of deletion (4 bp and 8 bp) in the 5′UTR. These observations could prove that these copies are not assembly artifact.

1. Introduction

Defensins are members of a cationic protein family (3–4 kDa) with six conserved cysteine residues that create three cysteine–disulfide bonds (Ganz and Lehrer, 1994). These conserved cysteines provide three different patterns for the disulfide bonds, that divide the defensin family into three subfamilies called α, β (Lehrer and Ganz, 1999), and θ-defensins (Tang et al., 1999). β-Defensins are in a single gene cluster in cattle and they were mapped to chromosome 27 (Gallagher et al., 1995). β-Defensin 103 (*DEFB103*) is a multi-functional gene influencing innate immunity (Bevins, 2006), coat color (Candille et al., 2007), and feeding behavior (Candille et al., 2007). All members of the β-defensin gene family in humans have two exons and one intron, with the exception of *DEFB105* (GenBank NM_152250.2) which has three coding exons and two introns (Pazgier et al., 2006).

Data from mRNA-seq showed that alternative splicing occurs in more than 90% of human genes (Pan et al., 2008; Wang et al., 2008). Genes with alternative promoter regions tend to generate alternative splicing (Xin et al., 2008). *Microphthalmia-associated transcription factor* (*MITF*) with nine exons (Tassabehji et al., 1994) and *insulin-like growth factor 2* (*IGF2*) with 10 exons (Goodall and Schmutz, 2007) are such genes. In most genes with alternative splicing, some of the exons (Goodall and Schmutz, 2007) or part of the exons (Udono et al., 2000) are non-coding.

β-Defensins are variable in their copy number (Hollox et al., 2003). *DEFB103* copy number variation has been reported in humans (2–12 copies per diploid genome) (Hollox et al., 2003) and dogs (2–4 copies per diploid genome) (Leonard et al., 2012). Cattle *DEFB103* occurs in multiple copies in at least some individuals, based on sequence results from a cloning experiment (GenBank KM347983, KM347984, KM347985, and KM347986). Five SNPs were reported in the 5′UTR of the *DEFB103* by Dreger and Schmutz (2009) (c.−319A>G, c.−264C>T, c.−69A>G, c.−42A>G, and c.−34G>A), and two additional SNPs (c.−383A>G, c.−241G>A) were identified in the 5′UTR of the *DEFB103* (Mirabzadeh-Ardakani et al., in press). The current research was conducted to investigate gene structure and haplotype variation of *DEFB103* in different tissues collected from dairy and beef cattle.

2. Materials and methods

2.1. Sample collection

Animals used for this study were humanely killed according to Canadian Council of Animal Care guidelines and protocols approved by the University of Saskatchewan Animal Care Committee. Tissue samples were collected from seven neonatal Holstein calves and five 6–8 month old Angus-cross beef calves. The tongue, buccal epithelium, conjunctiva, rumen, skin, trachea, and liver from neonatal calves and

Abbreviations: DEFB103, β-defensin 103; mRNA-seq, messenger ribonucleic acid sequencing; MITF, microphthalmia-associated transcription factor; IGF2, insulin-like growth factor 2; cDNA, complementary deoxyribonucleic acid; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; UTR, untranslated region; SNP, single nucleotide polymorphism; CNV, copy number variation.

* Corresponding author at: Department of Animal and Poultry Science, University of Saskatchewan, 51 Campus Dr., Saskatoon, SK S7N 5A8, Canada.
E-mail address: a.mirabzadeh@usask.ca (A. Mirabzadeh-Ardakani).

http://dx.doi.org/10.1016/j.gene.2014.08.055
0378-1119/

Appendix J: Manuscript published in *Cell and Tissue Research journal*. Mirabzadeh-Ardakani, A., Solie, J., Gonzalez-Cano, P., Schmutz, S.M. and Griebel, P.J. Tissue- and age-dependent expression of the bovine DEFB103 gene and protein. *Cell Tissue Res* (2015).

Cell Tissue Res
DOI 10.1007/s00441-015-2258-9

REGULAR ARTICLE

Tissue- and age-dependent expression of the bovine *DEFB103* gene and protein

Ali Mirabzadeh-Ardakani[1] · Jay Solie[2] · Patricia Gonzalez-Cano[2] · Sheila M. Schmutz[1] · Philip J. Griebel[2,3]

Received: 9 December 2014 / Accepted: 13 July 2015

Abstract Beta-defensin 103 (DEFB103) shares little homology with 8 other members of the bovine beta-defensin family and in other species DEFB103 protein has diverse functions, including antimicrobial activity, a chemoattractant for dendritic cells, enhancing epithelial wound repair and regulating hair colour. Expression of the bovine *DEFB103* gene was surveyed in 27 tissues and transcript was most abundant in tissues with stratified squamous epithelium. Oral cavity epithelial tissues and nictitating membrane consistently expressed high levels of *DEFB103* gene transcript. An age-dependent decrease ($P<0.05$) in *DEFB103* gene expression was only observed for buccal epithelium when comparing healthy 10- to 14-day-old and 10- to 12-month-old calves. A bovine herpesvirus-1 respiratory infection did, however, significantly ($P<0.05$) up-regulate *DEFB103* gene expression in the buccal epithelium of 6- to 8-month-old calves. Finally, *DEFB103* transcript was low in lymph nodes draining the skin and at the limit of detection in other internal organs such as lung, intestine and kidney. Affinity-purified rabbit antisera to bovine DEFB103 was used to identify cells expressing DEFB103 protein within tissues with stratified squamous epitheliums. DEFB103 protein was most abundant in basal epithelial cells and was present in these cells prior to birth. Beta-defensins have been identified as regulators of dendritic cell (DC) chemokine responses and we observed a close association between DCs and epithelial cells expressing DEFB103 in both the fetus and newborn calf. In conclusion, bovine *DEFB103* gene expression is most abundant in stratified squamous epithelium with DEFB103 protein localised to basal epithelial cells. These observations are consistent with proposed roles for DEFB103 in DC recruitment and repair of stratified squamous epithelium.

Keywords Basal epithelial cells · Beta-defensin 103 · Cattle · Dendritic cells · Stratified squamous epithelium

Electronic supplementary material The online version of this article (doi:10.1007/s00441-015-2258-9) contains supplementary material, which is available to authorized users.

✉ Ali Mirabzadeh-Ardakani
a.mirabzadeh@usask.ca

[1] Department of Animal and Poultry Science, University of Saskatchewan, 51 Campus Dr., Saskatoon, SK S7N 5A8, Canada

[2] Vaccine and Infectious Disease Organization/InterVac, University of Saskatchewan, Saskatoon, SK S7N 5E3, Canada

[3] School of Public Health, University of Saskatchewan, Saskatoon, SK S7N 5E3, Canada

Introduction

Antimicrobial peptides, also known as host defense peptides, are an important component of the innate immune system (Cederlund et al. 2011). They include several families of structurally diverse molecules, consisting in less than 100 amino acids and perform diverse functions, including both direct antimicrobial activity and modulation of innate immune functions (Ganz and Lehrer 1994). The expression of antimicrobial peptides is highest in tissues and cells that provide an important barrier to commensal and pathogenic microbes and this expression pattern is conserved among vertebrates, invertebrates and plants (Ganz 2003a, b).

Defensins are one family of antimicrobial peptides produced in mammals that share structurally similar motifs (Boman 1995). They are 3- to 4-kDa cationic proteins with six conserved cysteine residues that create three cysteine–disulfide bonds (Ganz and Lehrer 1994). Three different patterns of disulfide bonding among the conserved cysteines

Published online: 25 August 2015