CHARACTERIZATION OF THE BOVINE CATHELICIDIN GENE FAMILY

A Dissertation

by

ERIN GILLENWATERS FLORES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2011

Major Subject: Genetics

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Approved by:

Chair of Committee,	James E. Womack
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ABSTRACT

Characterization of the Bovine Cathelicidin Gene Family. (August 2011)

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Chair of Advisory Committee: Dr. James E. Womack

Cathelicidins (CATHLs) are small, cationic antimicrobial peptides that establish an early innate immune defense against infections in mammals. Beyond their wide spectrum of antimicrobial activity, these peptides play important roles in wound repair, chemotactic activity, and apoptosis. Thus, comprehensive characterizing of bovine CATHLs could potentially identify underlying inherited differences in innate immunity and disease resistance in cattle. The purpose of the present study was to verify the placement of the CATHL cluster at the distal end of bovine chromosome 22 (BTA22), identify any single nucleotide polymorphisms (SNPs) and insertion-deletion (indel) polymorphisms within the gene family, explore copy number variation, and investigate the functional impact any of these variants may have in overall bovine innate immunity.

A framework radiation hybrid map was constructed with 7 markers screened against the bovine 12,000 rad whole genome RH (12K WG-RH) panel, which when compared to the current genome assembly (Btau_4.0) confirmed current gene order. Comparative sequence analysis for 10 domestic cattle breeds representing both *Bos taurus taurus* and *Bos taurus indicus* revealed 60 SNPs, 7 of which were nonsynonymous, and 5 indel mutations. Data from array comparative genomic

hybridization (aCGH) between four Angus and four Nelore animals showed a 2-fold increase in copy number of the CATHL4 locus, which was verified by quantitative PCR (qPCR) of genomic DNA. Nelore animals showed an approximate 2-fold increase in the CATHL4 gene. Subsequently, the expression of CATHL4 in Nelore neutrophils exhibited a range of 2- to 5-fold increases in CATHL4 gene expression. Finally, a colorimetric bactericidal assay was performed on the neutrophils of the same Angus and Nelore animals previously genotyped for copy number variations (CNVs). After *in vitro* challenges to *Staphylococcus aureus*, *Salmonella typhimurium*, *Mannheimia haemolytica,* and *Pasteurella multocida*, the killing capacity of Nelore neutrophils was approximately 20% greater than Angus neutrophils for *M. haemolytica* and 10% greater for *P. multocida*. Characterization of this antimicrobial gene family is central to developing a firm understanding regarding the effects CATHL variation has with respect to bovine innate immunity.

DEDICATION

I would like to dedicate this manuscript to my husband Benjamin. Thank you for your love and support and for being my rock. It is also written in loving memory of my father, Richard Lawson Gillenwaters.

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I would like to thank my committee chair, Dr. Womack, for his guidance and support throughout the course of this research. His kind heart and great attitude have been wonderful sources of inspiration, both in science and in virtue. Thanks to my committee members, Dr. Derr, Dr. Long, and Dr. Riggs, for pushing me to be the best. Thanks also go to my friends and colleagues for making my time at Texas A&M University unforgettable. I also want to extend my gratitude to the Dindot, Cohen, and Lawhon labs. Without their hard work, dedication, and expertise many of these experiments would not have been possible. I sincerely appreciate Dr. Jason Sawyer for the use of the McGregor Research Facility's animals. Finally, thanks to my mother for her love and encouragement and to my husband for his patience.

NOMENCLATURE

CATHL	Cathelicidin
AMP	Antimicrobial Peptide
SNP	Single Nucleotide Polymorphism
Indel	Insertion/Deletion
RH	Radiation Hybrid
qPCR	Quantitative Real-Time PCR
CNV	Copy Number Variation
BRD	Bovine Respiratory Disease

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CHAPTER I

INTRODUCTION

Cattle

The book of Genesis tells the story of Abram and his journey into Egypt. Because of his beautiful wife, Sarai, Pharaoh found favor with Abram and blessed him with herds of sheep and cattle. For generations cattle represented sustenance, wealth, prosperity, and favor with God; they were offered up as burnt offerings and made to be false idols. In the millennia since Biblical times, cattle have played essential characters in the story of the human race.

Since biblical times, cattle have been domesticated for human use and therefore have become a research organism for agricultural, economic, scientific, and medical foci. Cattle belong to the taxonomic order Cetartiodactyl, represent Ruminantia, and occupy diverse environments worldwide, wherein they efficiently convert low-quality forage into energy-dense fat, muscle, and milk [1]. Human exploitation of these complex biological processes has resulted in over 800 breeds of domesticated cattle to date [1]. The intricate physiology of cattle and selective breeding has resulted in an organism which proves to be useful for studying the genetics of valuable agricultural traits, comparative genomics, and disease resistance.

This dissertation follows the style of Genomics.

Cattle can be separated into 2 geographic types, which diverged about 250,000 years ago: taurine (*Bos taurus taurus*), generally originating in Europe; and indicine (*Bos taurus indicus*), originating in East Africa and South Asia [2]. Both *Bos* subspecies include breeds suited for milk production and beef production, but there has been long-standing anecdotal evidence that *B. taurus indicus* animals weather harsh environmental conditions [3] and common pathogens [4] more efficiently than *B. taurus taurus* animals. Consequently, many American cattlemen take advantage of the hybrid vigor observed when indicine cattle are bred to taurine cattle. In 2009 the Bovine HapMap Consortium screened 14 taurine breeds (n=376), 3 indicine breeds (n=73), and 2 hybrid breeds (n=48) for SNPs in the search to uncover the genetic structure and history of the taurine and indicine subspecies [2]. Based on population mutation rates (θ) and pairwise nucleotide heterozygosity (π), taurine breeds showed lower nucleotide variation than indicine breeds, but both were higher than human populations [2]. The authors concluded that the taurine pre-domestication ancestral population was less diverse than the indicine ancestral populations, likely due to glacial habitat restrictions. Importantly, high degrees of differentiation based on average F_{ST} values were observed in genes associated with behavior, immunity, and feed efficiency [2].

Innate Immunity and Antimicrobial Peptides

Mammalian innate immunity provides a first line of defense against invading pathogens. It is activated immediately to rapidly control the replication of microbial invaders. Innate immunity can be classified into three kinds of barriers: anatomical

(skin, including interior epithelial layers, and its secreted chemicals), innate immune cells (granulocytic basophils, eosinophils, and neutrophils), and humoral (cellular messengers such as chemokines and cytokines) [5]. Pattern-recognition receptors (PRRs) are expressed on dendritic cells, B cells, and macrophages. These PRRs are encoded in the germline with predetermined specificity against conserved pathogen-associated molecular patterns (PAMPs) and often interact with antimicrobial peptides (AMPs) and other ligands [5]. The nature of the mode of action of the innate immunity is not to retain "memory" of specific antigens like the acquired immune system, but to provide consistent protection against a large pool of microbes based on their common PAMPs.

Antimicrobial peptides, or host defense peptides (HDPs) help to bridge the gap between innate and acquired immunity. They are expressed in epithelial cells and phagocytes, where they are stored in granules and released at mucosal surfaces and sites of infection [6]. Most are cationic and confer action against bacteria, viruses, fungi, and parasites. The main mode of microbicidal activity is disruption of the anionically charged membranes of pathogens. In addition to directly killing pathogens, AMPs also modulate other immune system functions like immune cell recruitment to sites of infection, tissue and wound repair stimulation, and pro-inflammatory cytokine suppression [6]. Two families of AMPs exist in mammals, the defensins and cathelicidins.

Cathelicidins

Members of the mammalian cathelicidin (CATHL) gene family are examples of the evolutionary processes that generate diverse molecules in regards to size, sequence, and structure, while maintaining beneficial antimicrobial properties [7]. Precursor CATHLs are found in myeloid cells, epithelial cells of the skin, oral mucosa, and the gastro-intestinal tract, while the mature forms of these antimicrobial peptides (AMPs) are found at mucosal surfaces and within bodily secretions like sweat, breast milk, and saliva [8]. Cathelicidins are cationic in nature, with a conserved N-terminal cathelin-like prosequence and a diverse C-terminal antimicrobial domain. While the prosequence (~100 residues in length) is conserved across many mammalian species, the antimicrobial domain is highly heterogeneous in both length (12-80 residues) and structure (α-helices, ß-hairpins stabilized by disulfide bonds, and specific amino acid-enriched structures) [9]. To date, components of the CATHLs have been identified in humans [10-12], monkeys [13, 14], mice [15, 16], rats [17], rabbits [18], guinea pigs [19], pigs [20-26], sheep [27, 28], goats [29], horses [30], dogs [31], and cattle [32-37]. Previous studies have established that humans and mice possess a single CATHL gene, whereas 3 CATHL loci are present within the horse genome. Moreover, both pigs and cattle have a large CATHL gene family comprising up to 11 members.

All CATHLs share a conserved four exon/three intron organization; the precursor cathelin-like domain is encoded in exons I-III, while the mature antimicrobial peptide, the cleavage site, and the 3' UTR are encoded in exon IV [7]. A net positive charge and the ability to fold into amphipathic structures enables mature cathelicidins to bind to

negatively charged microbial surfaces and to disrupt their membranes, resulting in rapid inactivation of the invading microbe [38]. However, it is now widely known that these molecules must be proteolytically processed in order to become active. Upon activation and degranulation of circulating neutrophils, the mature C-terminal antimicrobial peptides of mammalian CATHLs are released via a signal peptidase [39] (protease or elastase). Once cleaved and active, CATHLs have broad-spectrum microbicidal activity against bacteria, viruses, and fungi [40].

Several members of the bovine CATHL gene family are grouped in the linear α-helical structural class and show antimicrobial effectiveness against a wide variety of clinically relevant Gram-positive and Gram-negative bacteria [41]. Specifically, the bovine CATHL6 AMP demonstrated antimicrobial activity against *Pseudomonas aeruginosa* [42], and the bovine CATHL7 AMP completely inhibited the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) [43].

The single human cathelicidin LL-37 shares the linear α-helical structure with bovine CATHLs 5, 6, and 7, and exhibits antimicrobial activity against similar microbes. In recent years, widespread use of antibiotics has led to possible bacterial resistance, and natural antibiotics encoded by the CATHL genes may ultimately enable novel treatment strategies for resistant infections in both humans and animals. Recent human studies have focused on correcting the deficit of antimicrobial activity in the lungs of cystic fibrosis (CF) patients, who chiefly suffer from chronic inflammation and infection from *Pseudomonas aeruginosa*. Previous experiments showed that overexpression of LL-37 in a human bronchial xenograft model reversed the deficiency of bacterial killing

specific to CF [44]. The human and cattle genomes share approximately 70% sequence homology [8], so studying the underlying genetics of immunity could help us understand the extent of similarity between both organisms' innate immune responses.

The Bovine Immune System and Genetic Variation

Understanding bovine disease processes and incidence of susceptibility and resistance are of great economic concern worldwide. The health of herds in the beef and dairy industries remain a top priority for both cattlemen and researchers. Host-pathogen interactions vary widely among cattle, and it has been accepted that much of the variability in response to pathogens can be attributed to genetics. Additionally, differences in infectious disease resistance or susceptibility are observed both between breeds and within breeds. Traditional methods to control microbial infections include vaccination regimens and prophylactic and prescribed antibiotic and other pharmaceutical treatments. With a growing public concern for "chemical-free" beef products, many breeders are also incorporating animals with natural pathogen resilience to into their breeding programs [45]. A recent study focused on differences in *Bos* subspecies' resistance and susceptibility to internal parasites. When compared to *B. taurus indicus* animals, growth rates of *B. taurus taurus* animals are slowed when internal parasites are not controlled [45]. Importantly, taurine animals were shown to carry 75% more of an external parasite load than indicine crossbreeds in an Australian tick study [46]. These differences are observed between *B. taurus taurus* and *B. taurus indicus*, for which the very basis of difference is genetic makeup.

Uncovering genetic factors of infectious disease resistance and susceptibility will help achieve efficient and efficacious treatment regimens in today's cattle industry. In 2010, several immune-related genes of 380 Canadian dairy cattle were surveyed for SNPs associated with *Mycobacterium avium* spp. *paratuberculosis* (MAP) infection, also known as Johne's disease. Infection with MAP causes chronic inflammation in the gastrointestinal tract and shows similar symptoms to human irritable bowel disease (IBD). Sequence analysis revealed 4 SNPs in the ligand-binding subunit of interleukin-10 (IL10), IL10RA, which were strongly associated with MAP infection status [47]. Haplotype analysis of 3 of the 4 SNPs also showed strong association. Notably, these SNPs are synonymous or so-called "silent mutations." While these findings do not prove IL10RA SNPs are associated with resistance or susceptibility to MAP infection in dairy cattle, they provide a basis for future functional studies to reveal this host-pathogen pathway.

Bovine spongiform encephalopathy (BSE) is a prion disease characterized by neurodegenerative symptoms like dementia and ataxia. The spread of BSE is thought to occur through ingestion of infected meat or bone-meal and is transmissible to humans. In 2004 the bovine PRNP gene was screened for SNPs and indels in 55 animals of common German cattle breeds. A novel indel 23 base pairs in length was observed in the 5'-flanking sequence of PRNP [48]. The allelic and genotypic frequency was significantly different in healthy versus BSE-infected cattle, with the insertion found more frequently in healthy cattle [48]. The authors caution that limited sample number warrant further investigations.

Finally, a recent investigation of CNVs in Angus cattle by array comparative hybridization (aCGH) revealed CNVs in the MHC genes CIITA and ULBP, whose higher expression in mice previously conferred parasite resistance [49]. The authors postulate that increased diversity in major histocompatibility complex (MHC) Class II genes (like ULBP), combined with a set of alleles for efficient-presenting parasite antigen (CIITA) could help overall parasite resistance of the host [50]. The genomic variation of these immune system genes necessitates validation of their expression and functional implications.

CHAPTER II

HIGH-RESOLUTION RADIATION HYBRID MAP OF BOVINE CATHELICIDINS

Introduction

High resolution RH mapping still remains a valuable tool for building genomic maps, but more importantly, facilitates the validation of current genome assemblies [51]. Currently, the 12,000 rad whole genome RH (12K WG-RH) panel has been shown to have the highest mapping resolution for the bovine genome [52]. Since the start of the bovine sequencing project in 2003, several assemblies have been released. According to the Baylor College of Medicine Bovine Genome Project, the latest assembly, Btau_4.0, was released in October 2007, offered 7.1x coverage and was constructed by a combination of whole genome shotgun reads and BAC end sequences. Subsequently, it has been supplemented with more complete sequence data and Btau_4.2 was released in April 2009. Despite the many revolutions of the assembly, many discrepancies in gene order remain, and the region of the CATHL loci on BTA22 seems to be structurally complex at best.

Previous studies tentatively localized 6 phage clones containing five CATHL genes and one CATHL pseudogene to bovine chromosome 22q24 via fluorescence in situ hybridization (FISH) [53]. The authors observed several hybridization spots for some of the clones, indicating possible gene duplications, and suggested finer mapping was required to firmly establish gene order. Here, the 12K WG-RH panel confirms the overall gene order for this closely related gene family.

Materials and Methods

Primer Design

Based on available sequence data from the Baylor College of Medicine Bovine Genome Project Btau_4.0 genome assembly, primers were designed for as many unique CATHL loci as possible using Primer3 online (http://frodo.wi.mit.edu/) with optimal annealing temperature set at 58°C. Comprehensive searches of the annotated genome (NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and UCSC (http://genome.ucsc.edu/cgi-bin/hgGateway) nucleotide alignment tools) revealed the sequences of one pseudogene and one duplicated gene in the CATHL region. The currently annotated CATHL genes, the pseudogene, the duplicated gene, and three STS markers (UniSTS277967, UniSTS 268269, and UniSTS255316) were included in the analysis. Primers for CATHLs 1, 2, 3, 4, 5, 6, 7, pseudoCATHL, and DuplicateCATHL4 targeted the 3' end of the gene sequence to ensure specificity. UCSC's *in-silico* PCR function (http://genomemirror.duhs.duke.edu/cgi-bin/hgPcr?command=start) was employed to validate all primer pairs and the resultant amplicon sequences were BLATed in the bovine genome for further verification.

Genotyping of Selected Markers

PCR typings were performed in duplicate in 10 µl volumes. Working stocks of all primers were diluted to 10 µM for PCR amplification. Thermocycling parameters were as follows: 5 m at 95°C; 30 s at 95°C, 20 s at 55-65°C, and 30s - 2m at 72°C for 35 cycles, 10 m at 72°C. Each 10-µl PCR contained the following: 50 ng DNA, 0.2

mM each dNTP, 0.4 μM each primer (forward and reverse), 1.5 mM MgCl2, 1X GeneAmp PCR Gold Buffer (Applied Biosystems, Foster City, CA, USA), 0.2X MasterAmp PCR Enhancer (Epicentre, Madison, WI, USA), and 3 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). All resulting PCR products were visualized via ethidium bromide-stained agarose gel (2.0%) electrophoresis. All markers were genotyped at least twice, with positive (bovine genomic DNA), negative (rodent genomic DNA), and no template controls.

RH Map Construction

Radiation hybrid maps were constructed using the software package CarthaGene [54]. The program builds maximum multipoint likelihood consensus maps for which specifically optimized algorithms for RH data is available. Group distance and LOD were set to 0.0 and 7.0, respectively. The `nicemapl` and `nicemapd` commands quickly provide maps based on 2-points LOD criteria and 2-points distances criteria, and both were applied to the data set. These initial maps were enhanced with the default `flips` command, followed by the default `polish` command. Due to numerous marker order inaccuracies compared to the current Btau_4.0 assembly, a framework map was built. Using default parameters, the command `buildfw` was applied to the dataset, followed by default `flips` and default `polish`. Six markers were dropped in the construction of this map, so the same framework process was applied again, but with LOD thresholds of 1.0, where 7 markers were retained.

Summary of Experimental Results

The bovine 12,000 rad whole genome RH (12K WG-RH) panel was used to validate and complement existing assemblies of the CATHL gene cluster on BTA22q24. CATHL-specific markers were designed for all annotated CATHL genes and an additional pseudogene and duplicated gene. Three additional STS markers were included and all predicted marker amplicons were confirmed by UCSC's BLAT algorithm for specificity. The average retention frequency was 12.0 and the total map length was 126.1 cR,12000. Figure 1 shows marker retention frequencies and Figure 2 shows framework RH maps constructed using the CarthaGene software package [54] compared to the distal end of BTA22 according to the current bovine build Btau_4.0.

Initial maps were constructed with all markers, but discrepancies were found when compared to established marker order according to the current genome assembly; therefore framework maps were constructed. When LOD thresholds of 3.0 were applied, 6 of the 12 markers were retained on the map: UniSTS277967, CATHL4, DuplicateCATHL4, CATHL6, UniSTS268269, and UniSTS255316. The CATHL*5* marker was added to the framework map when a LOD threshold of 1.0 was applied. All maps were refined and confirmed with the `flips` command (all possible permutations with a sliding window of 5) followed by the `polish` command (displaces each marker in all possible intervals).

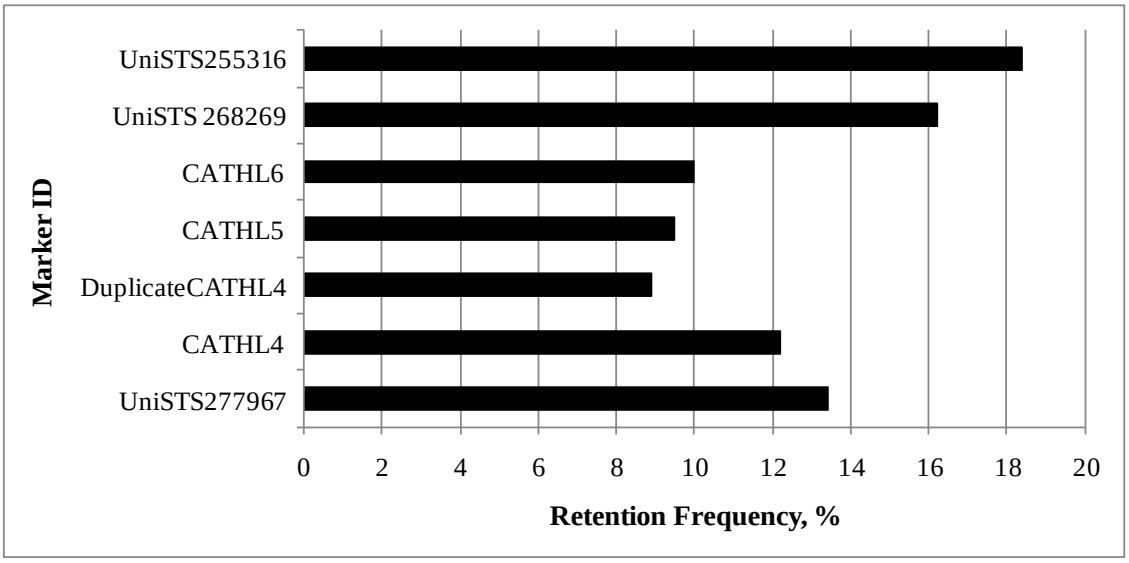

Fig. 1. Retention frequencies of markers on BTA22q24. Markers were genotyped and used to build framework radiation hybrid maps of the bovine CATHL cluster at the distal end of BTA22.

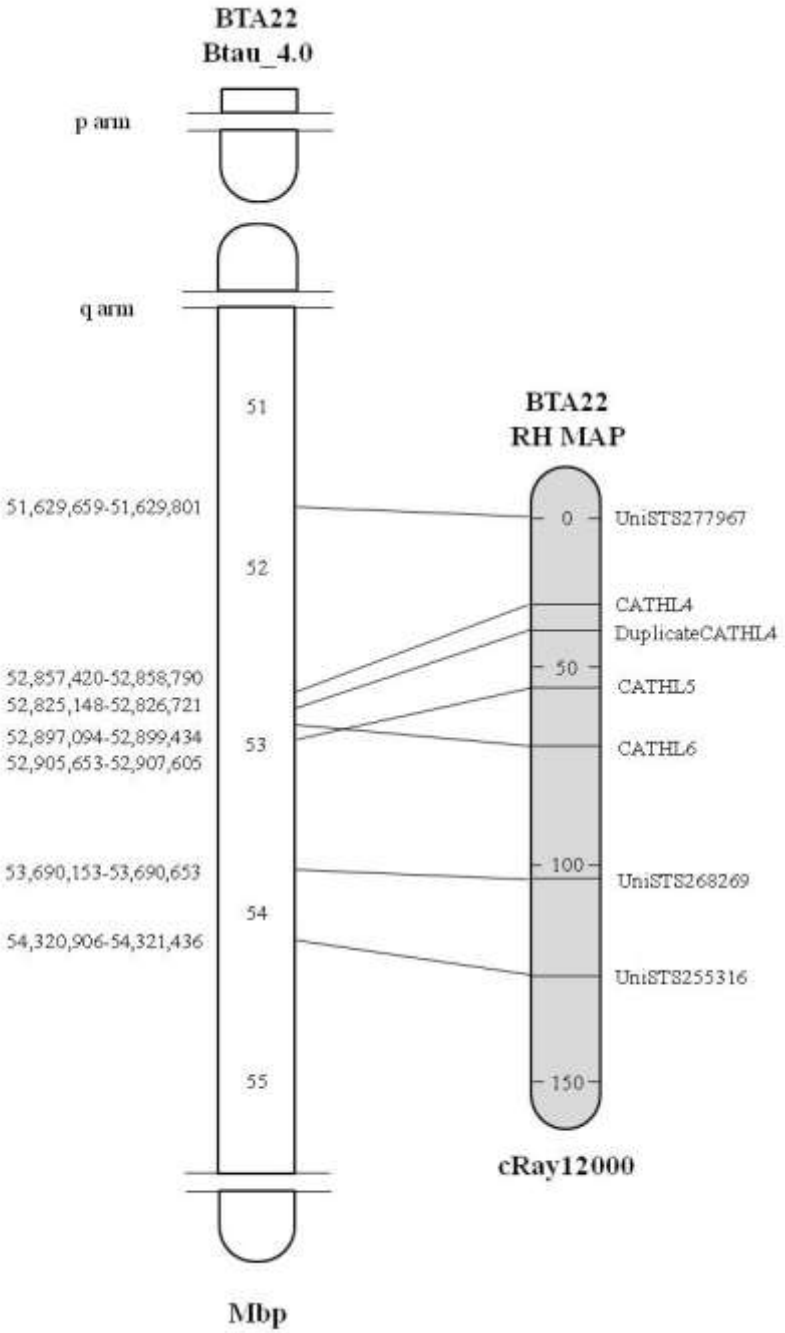

Fig 2. RH map of BTA22 compared with the corresponding bovine build Btau_4.0. The lines between maps connect markers on both maps. Distances of the RH map (right) are scaled in CentiRays (cR) and on the bovine build (left) Btau_4.0 in megabase (Mbp) pairs. Exact bp position for each marker (far right) is given on the far left.

Discussion

The bovine 12,000 rad whole genome RH (12K WG-RH) panel was screened with 12 markers which mapped to the distal end of BTA22 according to the bovine genome assembly Btau_4.0. In a previous study, 6 phage clones containing 5 CATHL genes and one CATHL pseudogene were localized to bovine chromosome 22q24 via fluorescence in situ hybridization (FISH) [53]. When initial maps were constructed with CarthaGene software, differences were detected when compared to the Btau_4.0 assembly, so framework maps were constructed. While the high radiation dose used in the construction of the 12K WG-RH panel generally provides quite high resolution of marker orders, the power is likely unable to resolve markers that are within a few Kb of each other. In the final framework map, one small inversion was detected between CATHL5 and CATHL6, likely attributed to the close proximity of the bovine CATHLs in the genome and the low LOD score (1.0) parameters. Initially, a LOD of 3.0 was applied, and the CATHL5 marker did not appear on the map, suggesting the likelihood of the inversion observed between markers CATHL5 and CATHL6 to be low. However, on both the preliminary framework map (LOD 3.0) and the final map (LOD 1.0) one marker associated with an apparent duplicated copy of the CATHL4 gene was firmly placed in order according to the current Btau_4.0 assembly. This helps verify the physical complexity of this gene family, and the RH map constructed here supports the overall structure of the current bovine genome assembly and previous FISH mapping. Given the small region this RH map encompasses, further verification of the bovine CATHL gene family order could be quickly accomplished with high-throughput

sequencing technologies or with Sanger sequencing. The advantage of high-throughput sequencing could be the independence from any previously known knowledge of CATHL gene order or current annotation status. Sanger sequencing is simpler and does not pose the same technical challenge that high-throughput sequencing does, but is more time consuming. Regardless, careful sequencing of the whole CATHL cluster on BTA22 would be beneficial.

CHAPTER III

SEQUENCE ANALYSIS AND POLYMORPHISM DISCOVERY*

Introduction

Cathelicidins of human and bovine origin are diverse but share conserved functions [8], a major factor influencing our decision to study the bovine CATHL genes. While the CATHL peptides exhibit broad-spectrum microbicidal activities, limited data currently exists with respect to the frequency and distribution of CATHL genetic polymorphisms. We considered CATHLs to be potentially important candidate genes for disease resistance studies in both humans and cattle. Herein, we examined genetic diversity within four members of the bovine CATHL gene family, CATHLs 2, 5, 6, and 7 located on *B. taurus taurus* chromosome (BTA22). A bovine DNA panel consisting of nine domestic cattle breeds representative of *B. taurus taurus*, *B. taurus indicus*, and composite breeds (*B. taurus taurus* - Angus, Charolais, Holstein, Limousin; *B. taurus indicus* - Brahman, Nelore; composite - Braford, Piedmontese, Romagnola) was utilized to facilitate comparative sequence analyses for four bovine CATHL genes. The Hereford reference sequence from the Bovine Genome Project (http://www.hgsc.bcm.tmc.edu/projects/bovine/) was also included. The origins of the aforementioned breeds were derived from the Oklahoma State University cattle breeds website (http://www.ansi.okstate.edu/breeds/cattle/).

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*This chapter is reprinted with permission from Sequence variability and polymorphism discovery in 4 members of the bovine cathelicidin gene family by E.N. Gillenwaters, C.M. Seabury, J.S. Elliott, E.K. Owens, and J.E. Womack. J Hered, 2(2009) 241-245.

The results of this study provide novel genetic variants that can enable future case-control studies and functional assays designed to elucidate whether CATHL variation may potentially underlie inherited differences in bovine innate immunity.

Materials and Methods

Primers and PCR amplification

All PCR primers were designed using Primer3 online (http://frodo.wi.mit.edu/) with optimal annealing temperature set at 58°C. Primer pairs were designed from the Btau_3.1 assembly but verified for specificity with the Baylor Btau_4.0 draft assembly (http://www.hgsc.bcm.tmc.edu/projects/bovine/) using UCSC's In-Silico PCR (http://genomemirror.duhs.duke.edu/cgi-bin/hgPcr?command=start), and by aligning the reference sequences for all CATHL genes and identifying primer sites. Primers within flanking regions were utilized to ensure gene-specific amplification. PCR primers for amplification and direct sequencing of CATHLs 2, 5, 6, and 7 are presented in Table 2. In total, two primer pairs were designed for PCR amplification of CATHL2, two pairs for CATHL5, three pairs for CATHL6, and one pair for CATHL7. Because the size of the amplicons generated for the CATHLs averaged ~2,500 nucleotides in length, multiple forward internal sequencing primers were designed to facilitate complete sequencing coverage. Working stocks of all primers were diluted to 10 μM for PCR amplification and direct sequencing. To generate an amplicon for CATHL5, a single thermocycling procedure was used in conjunction with 50 μl reaction volumes as follows: 5 m at 95°C; 30 s at 95°C, 20 s at 61°C, and 2 m at 72°C for 5 cycles; 30 s at

95°C, 20 s at 59°C, and 2 m at 72°C for 45 cycles, 10 m at 72°C. CATHL2 amplicons were generated via the following parameters: 5 m at 95°C; 30 s at 95°C, 20 s at 62°C, and 2 m at 72°C for 8 cycles; 30 s at 95°C, 20 s at 60°C, and 2 m at 72°C for 35 cycles, 10 m at 72°C. Optimal thermal parameters for CATHL6 were as follows: E1 primers, 5 m at 95°C; 30 s at 95°C, 20 s at 65°C, and 45 s at 72°C for 45 cycles; CATHL6 Gap, 5 m at 95°C; 30 s at 95°C, 20 s at 63°C, and 1 m at 72°C for 5 cycles; 30 s at 95°C, 20 s at 61°C, and 1 m at 72°C for 40 cycles, 10 m at 72°C; CATHL6 E4, 5 m at 95°C; 30 s at 95°C, 20 s at 60°C, and 1 m at 72°C for 5 cycles; 30 s at 95°C, 20 s at 58°C, and 1 m at 72°C for 35 cycles, 10 m at 72°C. Optimal thermal parameters for CATHL7 were as follows: 5 m at 95°C; 30 s at 95°C, 20 s at 61°C, and 2 m at 72°C for 5 cycles; 30 s at 95°C, 20 s at 60°C, and 2 m at 72°C for 45 cycles, 10 m at 72°C. Each 50-µl PCR contained the following: 125-250 ng DNA, 0.2 mM each dNTP, 0.4 µM each primer (forward and reverse), 1.5 mM MgCl2, 1X GeneAmp PCR Gold Buffer (Applied Biosystems, Foster City, CA, USA), 0.2X MasterAmp PCR Enhancer (Epicentre, Madison, WI, USA), and 3 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). All resulting PCR products were visualized via agarose gel (1.5%) electrophoresis and then purified using the QIAquick PCR Purification Kit (QiaGen, Inc., Valencia, CA, USA) according to the manufacturer's protocol.

Sequencing

A control sample was used to verify CATHL sequence identities prior to the polymorphism screen. Thereafter, all purified bovine CATHL amplicons were directly

sequenced in both directions using Big Dye Terminator Cycle 291 Sequencing technology in conjunction with GeneAmp 9700 PCR Systems (Applied Biosystems). Each 10 µl sequencing reaction contained the following: 2 µl of BigDye v1.1 (Applied Biosystems), 2 µl of halfBD (Genetix USA, Inc., Boston, MA, USA), 1 µl of PCR primer (10 µM), 0.5 µl of MasterAmp, approximately 6 ng/100 bp of purified PCR product, and enough Gibco distilled water (Invitrogen, Grand Island, NY, USA) to bring the total volume to 10 µl. Thermocycling parameters for PCR product sequencing were as follows: 5 minutes at 95°C; 30 seconds at 95°C, 20 seconds at 50°C, and 4 minutes at 60°C for 50 cycles. Sequencing reaction products were purified using G-50 Sephadex columns (Biomax, Odenton, MD, USA) according to the manufacturer's recommendations, then dried using a SpeedVac and stored at -20°C. Samples were rehydrated with 15 µl of ABI HiDi Formamide (Applied Biosystems) and resolved on an ABI 3130 automated sequencer (Applied Biosystems).

DNA Samples

DNA from an Angus bull (JEW38) was used for initial primer optimization and verification of amplicon sequence identities via BLAST (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/) and/or CLUSTALW (http://align.genome.jp/) alignment with the corresponding Hereford reference sequence as previously described [55]. DNA for the Angus bull as well as nine additional cattle breeds was available in a local repository [55, 56]. In addition to the Angus sample (JEW38), one bovine DNA sample was selected from the following breeds: Braford, Brahman, Charolais, Holstein, Limousin,

Nelore, Piedmontese, and Romagnola. The source of the DNA was commercially available spermatozoa [56]. All CATHL sequences generated in this study were aligned to the Hereford reference sequence from the Baylor Bovine Genome Project (http://www.hgsc.bcm.tmc.edu/projects/bovine/) Btau_4.0 draft assembly using CLUSTALW online (http://align.genome.jp/).

Comparative Sequence Analysis

All bovine CATHL sequences were assembled within the program Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI, USA) using default assembly parameters. Sequence quality was assessed by manual examination of electropherograms, and subsequently verified by Sequencher 4.7 quality analysis score. All heterozygous nucleotides were marked manually with the appropriate IUPAC-IUB codes for heterozygosity during initial electropherogram inspection and confirmed by alignment of multiple overlapping sequences within Sequencher 4.7. Ambiguous polymorphisms were validated by a secondary round of PCR and direct sequencing, with the resulting consensus sequences exported for further analysis. The online utility SMART (http://smart.embl-heidelberg.de/) was used for prediction of bovine CATHL protein domain architectures. The normal mode of SMART was employed in conjunction with the following settings: Include PFAM domains, signal peptides, internal repeats, and intrinsic protein disorder. Additionally, SMART was utilized to investigate how predicted amino acid replacements influence the prediction of protein domain architectures as previously described [55, 57]. Predicted amino acid replacements were

sequentially evaluated in SMART according to how they were observed in the sample (genotypically) [55, 57].

Statistical Analysis

Two-tailed Fisher's exact test was performed using eXactoid web-based statistics software (http://www.exactoid.com/fisher/index.php), where $p<0.05$ was considered statistically significant.

Summary of Experimental Results

Comparative sequence analysis of CATHLs 2, 5, 6, and 7 for ten domestic cattle breeds revealed 60 SNPs and five indel mutations. Notably, all polymorphisms localized to exons I-III and introns I and II, but not to exon IV. Collectively, 75% (n = 45) of the SNPs detected were transitions and 25% (n = 15) were transversions. No putative CpG islands were detected by either CpGPlot (http://www.ebi.ac.uk/emboss/cpgplot/) or CpGProD (http://pbil.univlyon1.fr/software/cpgprod_query.html) online for any of the bovine CATHL genes investigated in this study. Predicted amino acid replacements encoded by nonsynonymous SNPs within bovine CATHL2, but not CATHLs 5, 6, or 7, resulted in protein domain alterations predicted by the simple modular architecture research tool (SMART) online (http://smart.embl-heidelberg.de/). Repetitive sequences were detected within the targeted region of bovine CATHLs 2, 5, 6, and 7 using RepeatMasker (http://www.repeatmasker.org). RepeatMasker is an online tool that screens DNA sequences for interspersed repeats and other low complexity

DNA elements. Common mammalian repetitive elements include short interspersed nucleotide repeats (SINEs), long interspersed nucleotide repeats (LINEs), medium reiteration repeats (MER1), and mammalian-wide interspersed repeats (MIRs). Each of these classifications was detected in the sequences generated here.

CATHL2

Comparative sequence analysis of 1,559 bp spanning the coding and contiguous flanking regions of CATHL2 for ten bovine breeds yielded 5 SNPs, one of which was nonsynonymous (Table 1). Three repetitive sequences totaling 353 bp were detected within the targeted region of bovine CATHL2: DNA/MER1_type (813-973), SINE/MIR (1017-1098), and simple repeat (1253-1362). Prediction of CATHL2 protein domain architectures via SMART online for *Bos taurus taurus* and *Bos taurus indicus* revealed a conserved signal peptide domain (amino acids 1-29) and a conserved Pfam Cathelicidin domain (amino acids 31-97). Notably, the single predicted amino acid replacement within bovine CATHL2 (66 Asp → Asn) resulted in SMART prediction of a Cystatin-like domain (amino acids 18-125). This nonsynonymous SNP (nsSNP) was observed in two composite cattle breeds, Piedmontese and Romagnola (Table 1).

Table 1.

Single nucleotide polymorphisms (SNPs) detected in bovine CATHL2 by comparative sequence analysis of ten domestic cattle breeds [a] and the corresponding GenBank accession numbers for the CATHL2 sequences generated.

Alleles[b]	**Genomic Pos.** [c]	**Observed Freq.**[d]	**Amino Acid Pos.**[e]	**Amino Acid**[f]	**(SNP Type) Breeds**[g]	**dbSNP ID**
G/A	432	0.90/0.10	66	**Asp/Asn**	(R) Ro, P	ss104806979
T/C	1355	0.90/0.10	N/A	N/A	(Y) Bn, L	ss104806980
C/G	1382	0.90/0.10	N/A	N/A	(S) Bn, L	ss104806981
G/A	1393	0.90/0.10	N/A	N/A	(R) Bn, L	ss104806982
G/A	1397	0.90/0.10	N/A	N/A	(R) Bn, L	ss104806983

Breed, GenBank	**Breed, GenBank**	**Breed, GenBank**
Angus, EU380697	Charolais, EU380691	Nelore, EU380694
Braford, EU380689	Holstein, EU380692	Piedmontese, EU380695
Brahman, EU380690	Limousin, EU380693	Romagnola, EU380696

[a] An, Angus; Bd, Braford; Bn, Brahman; Ch, Charolais; Hn, Holstein; L, Limousin; N, Nelore; P, Piedmontese; R, Romagnola. All bovine CATHL2 sequences are compared to the Hereford CATHL2 sequence (GenBank Accession No. NC_007320.2, NM_174826).

[b] Major/minor alleles are shown, with Hereford (NC_007320.2, NM_174826) allele shown as bold and underlined.

[c] Genomic positions based upon J.E.W. 38 Angus bull CATHL2 GenBank Accession No. EU380697.

[d] Observed frequencies for major and minor alleles, respectively.

[e] Amino acid position based upon Hereford GenBank Accession No. NC_007320.2, NM_174826.

[f] Amino acid(s) encoded by major and minor alleles, respectively. Predicted replacements are shown in bold.

[g] Heterozygous SNPs depicted using IUPAC heterozygosity codes. Homozygous SNPs depicted using single standard nucleotides. The breed abbreviations following the SNP type deviate from the Hereford GenBank Accession No. NC_007320.2, NM_174826.

CATHL5

Comparative sequence analysis of 1,875 bp spanning the coding regions of CATHL5 for a panel of ten cattle breeds revealed 18 SNPs. Of the 8 SNPs detected within the coding region of CATHL5, 2 were nonsynonymous.

Collectively, three repetitive sequences were detected, totaling 298 bp: LINE/L2 (907-989), DNA/MER1_type (1615-1748), and SINE/MIR (1781-1861). One of the SNPs detected for bovine CATHL5 occurs within the LINE/L2 described above (Table 2).

Prediction of bovine CATHL5 protein domain architectures using SMART online revealed a conserved signal peptide domain (amino acids 1-29) and a conserved Pfam Cathelicidin domain (amino acids 31-98). Predicted amino acid replacements encoded by bovine CATHL5 nsSNPs (Table 2) did not result in the prediction of novel protein domain architectures or protein domain alterations using SMART online.

Table 2.
Single nucleotide polymorphisms (SNPs) detected in bovine CATHL5 by comparative sequence analysis of ten domestic cattle breeds [a] and the corresponding GenBank accession numbers for the CATHL5 sequences generated.

Alleles[b]	**Genomic Pos.**[c]	**Observed Freq.**[d]	**Amino Acid Pos.**[e]	**Amino Acid**[f]	**(SNP Type) Breeds**[g]	**dbSNP ID**
A/G	47	0.90/0.10	11	Gly/Gly	(R) L, N	ss104806984
G/C	50	0.95/0.05	12	Arg/Arg	(S) L	ss104806985
C/T	82	0.90/0.10	23	**Ala/Val**	(Y) L, N	ss104806986
C/G	84	0.90/0.10	24	Leu/Leu	(S) L, N	ss104806987
C/G	182	0.95/0.05	56	Arg/Arg	(S) N	ss104806988
T/A	203	0.95/0.05	63	Pro/Pro	(W) L	ss104806989
C/A	246	0.90/0.10	N/A	N/A	(M) L, Ro	ss104806990
C/**T**	296	0.20/0.80	N/A	N/A	(Y) P, L; (C) N	ss104806991
C/**T**	300	0.20/0.80	N/A	N/A	(Y) P, L; (C) N	ss104806992
T/A	322	0.90/0.10	N/A	N/A	(W) L, N	ss104806993
G/C	326	0.90/0.10	N/A	N/A	(S) L, N	ss104806994
T/C	641	0.90/0.10	N/A	N/A	(Y) N, Bn	ss104806995
A/G	657	0.90/0.10	N/A	N/A	(R) N, Bn	ss104806996
T/C	691	0.90/0.10	N/A	N/A	(Y) N, Bn	ss104806997
C/T	704	0.90/0.10	N/A	N/A	(Y) N, Bn	ss104806998

Table 2 (cont'd)

Alleles[b]	Genomic Pos.[c]	Observed Freq.[d]	Amino Acid Pos.[e]	Amino Acid[f]	(SNP Type) Breeds[g]	dbSNP ID
C/T	707	0.90/0.10	N/A	N/A	(Y) N, Bn	ss104806999
T/C	921	0.90/0.10	93	**Ser/Pro**	(Y) N, Bn	ss104807000
C/T	1169	0.85/0.15	121	Phe/Phe	(Y) L, N, Ro	ss104807001

Breed, GenBank	Breed, GenBank	Breed, GenBank
Angus, EU751302	Charolais, EU751296	Nelore, EU751299
Braford, EU751294	Holstein, EU751297	Piedmontese, EU751300
Brahman, EU751295	Limousin, EU751298	Romagnola, EU751301

[a] An, Angus; Bd, Braford; Bn, Brahman; Ch, Charolais; Hn, Holstein; L, Limousin; N, Nelore; P, Piedmontese; R, Romagnola. All bovine CATHL5 sequences are compared to the Hereford CATHL5 sequence (GenBank Accession No. NC_007320.2, NM_174510).

[b] Major/minor alleles are shown, with Hereford (NC_007320.2, NM_174510) allele shown as bold and underlined.

[c] Genomic positions based upon J.E.W. 38 Angus bull CATHL5 GenBank Accession No. EU751302.

[d] Observed frequencies for major and minor alleles, respectively.

[e] Amino acid position based upon Hereford GenBank Accession No. NC_007320.2, NM_174510.

[f] Amino acid(s) encoded by major and minor alleles, respectively. Predicted replacements are shown in bold.

[g] Heterozygous SNPs depicted using IUPAC heterozygosity codes. Homozygous SNPs depicted using single standard nucleotides. The breed abbreviations following the SNP type deviate from the Hereford GenBank Accession No. NC_007320.2, NM_174510.

CATHL6

The total genomic size (including 956 bp of unresolved sequence data) of CATHL6 is 2,345 bp based on the current RefSeq (NC_007320.2, NM_174832.2). In this study we interrogated 2,052 bp of bovine CATHL6 and detected 28 SNPs and 5 indels. The entire coding region of CATHL6 was targeted, as well as 5' and 3'flanking sequences. Of the 9 SNPs identified within the CATHL6 coding region, 4 were nonsynonymous.

Following multiple sequence alignment, five indels were observed within intronic sequences located between exons III and IV of CATHL6. Notably, all nine breeds investigated share the same homozygous genotype for every indel observed, suggesting the possibility of sequence assembly errors associated with the Hereford CATHL6 reference sequence. According to the Hereford reference sequence, 957 bp of sequence data is currently unresolved in the intronic region between exons II and IV. Alignment between our fully sequenced individuals and the reference sequence via CLUSTALW (http://align.genome.jp/) indicates only a 537-bp gap exists, for which we have acquired complete sequence data. Consequently, this gap in the reference sequence includes the region which encodes CATHL6 exon III. However, the position and nucleotide sequence of this exon may be predicted based upon the high conservation of the bovine CATHL exons I-III.

Three repetitive sequences totaling 337 bp within the target region of CATHL6 were identified as follows: LINE/L2 (862-944), DNA/MER1_type (1551-1667), and

SINE/MIR (1683-1819). Altogether, 6 SNPs and 1 indel were detected within the three repeats identified by RepeatMasker (Tables 3 and 4).

Prediction of bovine CATHL6 protein domain architectures using SMART online revealed a conserved signal peptide domain (amino acids 1-29) and a conserved Pfam Cathelicidin domain (amino acids 31-98). Predicted amino acid replacements encoded by bovine CATHL6 nsSNPs (Table 3) did not result in the prediction of novel protein domain architectures or protein domain alterations using SMART online.

CATHL7

Based on the current RefSeq (NC_007320.2, NM_174831.1), CATHL*7* has a total genomic size of 1,871 bp. Comparative sequence analysis of 2,245 bp spanning the coding and contiguous flanking regions of bovine CATHL7 revealed 9 SNPs (Table 5).

RepeatMasker online revealed two repetitive sequences within CATHL7, totaling 216 bp, and contained the following repeats: LINE/L2 (645-723) and DNA/MER1_type (1344-1480).

All CATHL7 SNPs within the coding region were synonymous and did not result in predicted amino acid replacements. Therefore, SMART was not employed to investigate how predicted amino acid replacements influence the prediction of protein domain architectures.

Table 3.
Single nucleotide polymorphisms (SNPs) detected in bovine CATHL6 by comparative sequence analysis of ten domestic cattle breeds [a] and the corresponding GenBank accession numbers for the CATHL6 sequences generated.

Alleles[b]	**Genomic Pos.** [c]	**Observed Freq.**[d]	**Amino Acid Pos.**[e]	**Amino Acid**[f]	**(SNP Type) Breeds**[g]	**dbSNP ID**
C/T	490	0.95/0.05	N/A	N/A	(Y) N	ss104807002
C/T	494	0.85/0.15	N/A	N/A	(Y) N; (T) Bn	ss104807003
C/T	532	0.85/0.15	N/A	N/A	(Y) N; (T) L	ss104807004
T/G	562	0.85/0.15	N/A	N/A	(K) N; (G) L	ss104807005
C/G	812	0.75/0.25	N/A	N/A	(S) N; (G) Bn, L	ss104807006
C/T	930	0.85/0.15	N/A	N/A	(Y) N; (T) L	ss104807007
C/G	958	0.90/0.10	N/A	N/A	(G) Bn	ss104807008
A/G	959	0.90/0.10	N/A	N/A	(G) Bn	ss104807009
C/T	1090	0.75/0.25	84	Thr/Thr	(Y) N; (T) Bn, L	ss104807010
G/A	1093	0.75/0.25	85	Val/Val	(R) N; (A) Bn, L	ss104807011
G/A	1105	0.80/0.20	89	Thr/Thr	(A) N, L	ss104807012
G/C	1121	0.90/0.10	95	**Glu/Gln**	(C) Bn	ss104807013
A/G	1141	0.75/0.25	101	Glu/Glu	(R) N; (G) Bn, L	ss104807014
A/**T**	1144	0.10/0.90	102	**Lys/Asn**	(T) An, Bd, Bn, Ch, Hn, L, N, P, Ro	ss104807015
T/C	1185	0.75/0.25	N/A	N/A	(Y) N; (C) Bn, L	ss104807016

Table 3 (cont'd)

Alleles[b]	Genomic Pos.[c]	Observed Freq.[d]	Amino Acid Pos.	Amino Acid[f]	(SNP Type) Breeds[g]	dbSNP ID
C/T	1215	0.75/0.25	N/A	N/A	(Y) N; (T) Bn, L	ss104807018
A/G	1252	0.72/0.28	N/A	N/A	(R) N; (G) Bn, L	ss104807019
G/A	1330	0.67/0.33	116	Ala/Ala	(A) Bn, N, L	ss104807020
T/G	1345	0.67/0.33	121	**Ile/Met**	(G) Bn, N, L	ss104807021
G/A	1358	0.67/0.33	126	**Glu/Lys**	(A) Bn, N, L	ss104807022
G/A	1599	0.89/0.12	N/A	N/A	(A) Bn	ss104807023
C/T	1617	0.72/0.28	N/A	N/A	(T) Bn, N, L	ss104807024
G/A	1652	0.67/0.33	N/A	N/A	(A) Bn, N, L	ss104807025
G/**A**	1796	0.10/0.90	N/A	N/A	(A) An, Bd, Bn, Ch Hn, L, N, P, Ro	ss104807027
A/**C**	1798	0.10/0.90	N/A	N/A	(C) An, Bd, Bn, Ch, Hn, L, N, P, Ro	ss104807028
A/**G**	1835	0.10/0.90	N/A	N/A	(G) An, Bd, Bn, Ch, Hn, L, N, P, Ro	ss104807030
C/**G**	1856	0.10/0.90	N/A	N/A	(G) An, Bd, Bn, Ch Hn, L, N, P, Ro	ss104807031
T/**C**	1858	0.10/0.90	N/A	N/A	(C) An, Bd, Bn, Ch Hn, L, N, P, Ro	ss104807032

Breed, GenBank	**Breed, GenBank**	**Breed, GenBank**
Angus, EU751311	Charolais, EU751305	Nelore, EU751308
Braford, EU751303	Holstein, EU751306	Piedmontese, EU751309
Brahman, EU751304	Limousin, EU751307	Romagnola, EU751310

Table 3 (cont'd)

[a] An, Angus; Bd, Braford; Bn, Brahman; Ch, Charolais; Hn, Holstein; L, Limousin; N, Nelore; P, Piedmontese; R, Romagnola. All bovine CATHL6 sequences are compared to the Hereford CATHL6 sequence (GenBank Accession No. NC_007320.2, NM_174832).

[b] Major/minor alleles are shown, with Hereford (NC_007320.2, NM_174832) allele shown as bold and underlined.

[c] Genomic positions based upon J.E.W. 38 Angus bull CATHL6 GenBank Accession No. EU751311.

[d] Observed frequencies for major and minor alleles, respectively.

[e] Amino acid position based upon Hereford GenBank Accession No. NC_007320.2, NM_174832.

[f] Amino acid(s) encoded by major and minor alleles, respectively. Predicted replacements are shown in bold.

[g] Heterozygous SNPs depicted using IUPAC heterozygosity codes. Homozygous SNPs depicted using single standard nucleotides. The breed abbreviations following the SNP type deviate from the Hereford GenBank Accession No. NC_007320.2, NM_174832.

Table 4.

Insertion-deletion mutations (indels) observed during comparative sequence analysis of bovine CATHL6 for ten domestic cattle breeds [a] and the corresponding GenBank accession numbers for the CATHL6 sequences generated.

CATHL**6 indels**[b]	**bp**[c]	**Genomic Pos.**[d]	**Indel Sequence**	**Allele Freq.**[e]	**(Indel Genotype) Breeds**[f]	**dbSNP ID**
Deletion	3	1196	CAT	0.90	(-/-) An, Bd, Bn, Ch, Hn, L, N, P, Ro	ss104807017
Insertion	1	1784-1785	T	0.90	(+/+) An, Bd, Bn, Ch, Hn, L, N, P, Ro	ss104807026
Insertion	1	1799-1800	T	0.90	(+/+) An, Bd, Bn, Ch, Hn, L, N, P, Ro	ss104807029
Insertion	3	1858-1859	TTG	0.90	(+/+) An, Bd, Bn, Ch, Hn, L, N, P, Ro	ss104807033
Insertion	1	1925-1926	T	0.90	(+/+) An, Bd, Bn, Ch, Hn, L, N, P, Ro	ss104807034

[a] An, Angus; Bd, Braford; Bn, Brahman; Ch, Charolais; Hn, Holstein; L, Limousin; N, Nelore; P, Piedmontese; R, Romagnola. All CATHL6 sequences are compared to the Hereford CATHL6 sequence (GenBank Accession No NW_001494125, NM_174832.)

[b] Type of indel as compared to the Hereford CATHL6 GenBank Accession No. NC_007320.2, NM_174832.2.

[c] Number of base pairs inserted.

[d] Genomic positions based upon J.E.W. 38 Angus bull CATHL6 GenBank Accession No. EU751311.

[e] Observed frequency of the indel.

[f] Type of indel as compared to the Hereford CATHL6 GenBank Accession No. NC_007320.2, NM_174832.

Table 5.

Single nucleotide polymorphisms (SNPs) detected in bovine CATHL7 by comparative sequence analysis of 10 domestic cattle breeds [a] and the corresponding GenBank accession numbers for the CATHL7 sequences generated.

Alleles[b]	Genomic Pos.[c]	Observed Freq.[d]	Amino Acid Pos.[e]	Amino Acid[f]	(SNP Type) Breeds[g]	dbSNP ID
G/T	2	0.83/0.17	N/A	N/A	(T) N; (K) L	ss104807035
A/G	65	0.85/0.15	13	Ser/Ser	(G) N; (R) L	ss104807036
A/**G**	134	0.85/0.15	36	Glu/Glu	(A) N; (R) L	ss104807037
T/C	529	0.85/0.15	N/A	N/A	(C) N; (Y) L	ss104807038
G/T	592	0.85/0.15	N/A	N/A	(T) N; (K) L	ss104807039
T/C	644	0.85/0.15	N/A	N/A	(C) N; (Y) L	ss104807040
G/A	1039	0.85/0.15	N/A	N/A	(A) N; (R) L	ss104807041
C/T	1338	0.85/0.15	N/A	N/A	(T) N; (Y) L	ss104807042
G/A	1653	0.85/0.15	N/A	N/A	(A) N; (R) L	ss104807043

Breed, GenBank	Breed, GenBank	Breed, GenBank
Angus, EU380715	Charolais, EU380709	Nelore, EU380712
Braford, EU380707	Holstein, EU380710	Piedmontese, EU380713
Brahman, EU380708	Limousin, EU380711	Romagnola, EU380714

[a] An, Angus; Bd, Braford; Bn, Brahman; Ch, Charolais; Hn, Holstein; L, Limousin; N, Nelore; P, Piedmontese; R, Romagnola. All bovine CATHL7 sequences are compared to the Hereford CATHL7 sequence (GenBank Accession No. NC_007320.2, NM_174831).

[b] Major/minor alleles are shown, with Hereford (NC_007320.2, NM_174831) allele shown as bold and underlined.

[c] Genomic positions based upon J.E.W. 38 Angus bull GenBank Accession No. EU380715.

[d] Observed frequencies for major and minor alleles, respectively.

[e] Amino acid position based upon Hereford GenBank Accession No. NM_174831.

[f] Amino acid(s) encoded by major and minor alleles, respectively. Predicted replacements are shown in bold.

[g] Heterozygous SNPs depicted using IUPAC heterozygosity codes. Homozygous SNPs depicted using single standard nucleotides. The breed abbreviations following the SNP type deviate from the Hereford GenBank Accession No. NC_007320.2, NM_174831.

Discussion

Cathelicidins are small amphipathic peptides that exhibit rapid and potent activity against a wide variety of pathogens, including bacteria, viruses, and fungi [40]. In this study, we identified 60 SNPs and five indels for a panel of ten bovine breeds, including representatives of *Bos taurus taurus*, *Bos taurus indicus*, and composites thereof. The SNP density of these genes ranged from 1/73 bp (CATHL6) to 1/311 bp (CATHL2), with an average density of 1/128 bp. This density is comparable and not significantly different (two-tailed Fisher's exact test, P = 0.8299) to SNP densities for other bovine innate immunity genes investigated using the same bovine breed panel [57].

The single predicted amino acid replacement (66 Asp → Asn) identified within CATHL2 was observed in two composite breeds (Piedmontese, Romagnola) and results in prediction of an altered protein domain using SMART online (Pfam Cathelicidin domain → CY, Cystatin-like domain). This result is not surprising, given that the cathelin-like prosequence of the bovine CATHL genes show similarity to the cystatin superfamily (cysteine protease inhibitors) [38].

Using the online RepeatMasker software, the sequences generated here were screened for common mammalian repetitive sequences, including SINEs, LINEs, MER1 sequences, and MIRs. These repetitive sequences make up the majority of eukaryotic genomes, play a large role in evolution due to the tendency to cause insertions, deletions, and rearrangements, and influence nearby gene expression [58]. RepeatMasker is the most widely used tool for repetitive element discovery, but is limited by two factors: it only detects sequences similar to previously established repeat sequences; and it does

not discriminate between repetitive sequences found in multiple copies and genes found in multiple copies [58]. Therefore some of the repetitive sequences observed here could be due to repeated cellular genes or inherent limitations of the RepeatMasker algorithm.

The DNA used in this study was isolated from semen which was collected from a local commercial repository. Despite their breed classifications, it appears some of the bulls' genetic backgrounds may be more heterogeneous than their breeds would indicate. The Limousin bull used in this panel shared the same 4 heterozygous genotypes with the Brahman (*B. taurus indicus*) in CATHL2 (80% of total SNPs in CATHL2), 6 heterozygous genotypes with the Nelore (*B. taurus indicus*) and 4 heterozygous genotypes with the crossbreeds (44% of total CATHL5 SNPs), 7 homozygous genotypes with Brahman and 5 homozygous genotypes with both Brahman and Nelore in CATHL6 (46% of the SNPs found in CATHL6), and was heterozygous for every Nelore polymorphic site in CATHL7 (100% of CATHL7 SNPs). Only 2 SNPs in CATHL5 were specific to the Limousin and it never shared the same genotype as other *B. taurus taurus* breeds unless all animals in the panel shared the same genotype. With the exception of the Limousin, all SNPs reported here were detected in *B. taurus indicus* animals or crossbreeds (unless the whole panel shared the same genotype). While the number of samples in this study is quite limited, these results agree with findings of the Bovine HapMap Consortium that nucleotide diversity in immune system genes is higher in the *B. taurus indicus* subspecies [2]. According to high-resolution analysis of haplotype structure in cattle breeds, linkage disequilibrium and haplotype blocks range from 1-100 kb [59], and since the bovine CATHL locus spans about 100 kb, a *Bos*

subspecies-specific haplotype could exist for the CATHL gene cluster. Phase resolution together with a survey of SNPs in an expanded region around the CATHL locus could elucidate the haplotype structure of the CATHL genes. This could be informative for future searches for genetic variability and disease associations.

CHAPTER IV

COPY NUMBER VARIATION IN DOMESTIC CATTLE

Introduction

In recent years, the search for association between bovine infectious disease and genetic variation has focused on SNPs, indels, and microsatellites. These sources of genetic variation arise from changes in the DNA sequences, not overall structure. Concurrently, we have seen an expansion in interest from these direct changes in DNA sequence to structural changes which may confer a broader genetic impact: copy number variation (CNV). Copy number variants are defined as regions of DNA greater than 1,000 bp in length and are deleted or duplicated in a way that the region's copy number on either chromosome differs from 1 [60]. CNVs are thought to cover 12% of the human genome [61]. Notably, CNVs have very recently been recognized as a common source of inter-individual genomic variation and could play an important role in the adaptation and evolution to various environments in healthy individuals [61]. Historically, CNVs have been recognized as potential causes for human disease and in the last 5 years have been associated such common complex diseases as human immunodeficiency virus (HIV) [62], autoimmune diseases [63, 64], and several neuropsychiatric conditions [65]. These findings set the precedent for groundbreaking studies in other organisms and the search for disease association. To date, CNVs have been confirmed to exist in the genomes of chimpanzee [66, 67], rhesus macaque [68],

mouse [69-76], rat [77], fruit fly [78, 79], dog [80], chicken [81], pig [82, 83], goat [84, 85], and cattle [86-88].

Nearly a quarter of a century ago, reasonable suspicion existed that the CATHL gene family included duplicated gene copies [53]. Now, array comparative genomic hybridization (aCGH) offers a powerful and highly sensitive method of CNV detection along the genome. Copy numbers are calculated when raw data are normalized, aligned to their respective genomic locations, and then statistically analyzed to ensure reliability of detection [89]. The normalization process is imperfect and will not provide exact copy numbers, but changes in the normalized $\log_2$ ratios may reflect changes in true copy numbers [89]. It is imperative that aCGH results be further validated with a secondary method, usually quantitative real-time PCR (qPCR) or fluorescence in situ hybridization (FISH). Here, aCGH resulted in the detection of a CNV at the CATHL4 locus, and was confirmed with subsequent qPCR.

Materials and Methods

DNA Isolation

Whole blood was collected via jugular venipuncture from 8 animals maintained at the Texas A&M University Department of Animal Science McGregor Research Center (McGregor tag IDs: “704M,” “108M,” “183N,” “621N,” “356S,” “702P,” “707P,” and “770P”). Blood was stored in 8 mL EDTA vacutainers at 4°C to allow buffy coats to separate from red blood cells and plasma. DNA was isolated and purified from buffy coats using a standard phenol-chloroform protocol, as previously described

[90]. Genomic DNA from a bovine breed panel of 93 individuals was isolated from spermatozoa, commercially available at a local repository, as previously described [55].

Array Comparative Genomic Hybridization

A customized high-resolution bovine exon tiling microarray (Agilent Technologies) was developed previously by Dr. Scott V. Dindot and colleagues. The array contained oligonucleotide probes which were designed from the current Btau_4.0 assembly to cover the majority of the bovine exome. The DNA of one Angus and one Nelore animal were labeled with 2 different fluorophores and hybridized overnight in an Agilent Hybridization Oven, then washed and scanned on the SureScan Microarray Scanner (Agilent Technologies). Fluorescence intensities and $\log_2$ ratios were analyzed with the Genomic Workbench 5.0 software package (Agilent Technologies).

Quantitative Real-time PCR of Genomic DNA

Primers for CATHL4 targeted the 3' end of the gene sequence to ensure specificity. Primer pairs were designed using the default parameters of Biosearch Technology's RealTimeDesign software (http://www.biosearchtech.com/realtimedesign) and validated for specificity using the UCSC Genome Browser (http://genome.ucsc.edu/) set to the most current bovine genome assembly, Btau_4.2. SYBR Green MasterMix (Invitrogen) was used in conjunction with the ABI 7900HT Fast Real-Time PCR system for quantitative real-time PCR (qPCR) of the CATHL4 locus. Reactions were carried out in optical 384-well microplates (Bio-Rad) and contained the following: 1X final

concentration SYBR Green Master Mix, 200 nM final concentration forward and reverse primers, 25 ng DNA template, and Gibco distilled water to a final volume of 10 µl. Thermocycling parameters were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec, 60°C for 60 sec. Immediately following the last cycle a standard dissociation curve was performed to detect any primer-dimers in the PCR products. All PCR products were visualized by gel electrophoresis using 2% agarose gels stained with ethidium bromide. Initial C_T values of each reaction were verified to be within the logarithmic phase of the qPCR reaction. Using the relative comparative threshold cycle (C_T) method, duplicate C_T values were averaged and normalized against GAPDH for each assay [91]. Samples with C_T values that varied more than 0.5 were tested again. CATHL4 primers were: gCATHL4 F = 5'-GCAAAGGCTCAACCAGGAGT , gCATHL4 R = 5'-GGGTGTCCAGAAGAGACCAA) and GAPDH primers were GAPDH F = 5'-GAAGGTGAAGGTCGGAGTC, GAPDH R = 5'-GAAGATGGTGATGGGATTTC).

Summary of Experimental Results

Array Comparative Genomic Hybridization

Genomic DNA of 8 animals from the Texas A&M University Department of Animal Science McGregor Research Center were chosen to be screened for genomic structural variation. A customized high-resolution bovine exon tiling array (Doan *et al.*, unpublished data) was previously used to assess genomic structural variations of DNA from 4 Angus (McGregor Research Center tag IDs: "074M," "108K," "183N," "621N")

animals representing *B. taurus taurus*, and 4 Nelore (tag IDs: "356S," "702P," "707P," "770P") animals representing *B. taurus indicus*. Analysis of $\log_2$ ratios determined from hybridization signals indicated 2-fold higher fluorescence signals from the Nelore relative to the Angus at the CATHL4 locus and other biologically relevant loci. Figure 3 depicts the results of aCGH of 2 Angus (McGregor Research Center tag IDs: "108M," "183N") and 2 Nelore (tag IDs: "356S," "702P") over the CATHL4 gene on bovine chromosome 22q24.

Copy Number Variation of Angus and Nelore Cattle

Figure 4 contains the results of qPCR of CATHL4 in 4 Angus (McGregor Research Center tag IDs: "074M," "108K," "183N," "621N") and 4 Nelore ("356S," "702P," "707P," "770P") animals. GAPDH C_T values were subtracted from each sample C_T value (ΔC_T), which was then subtracted from the highest ΔC_T value ($\Delta\Delta C_T$). Fold difference was calculated as $2^{-(\Delta\Delta CT)}$. Based on average $2^{-(\Delta\Delta CT)}$ values for each breed, Nelore cattle showed an approximate 2-fold increase in relative copies of CATHL4 over the Angus breed. When an unpaired 2-tailed t-test compared Nelore fold differences to Angus fold differences, the breed was significantly different from Angus ($P=2.86E\text{-}4$, $P \leq 0.01$).

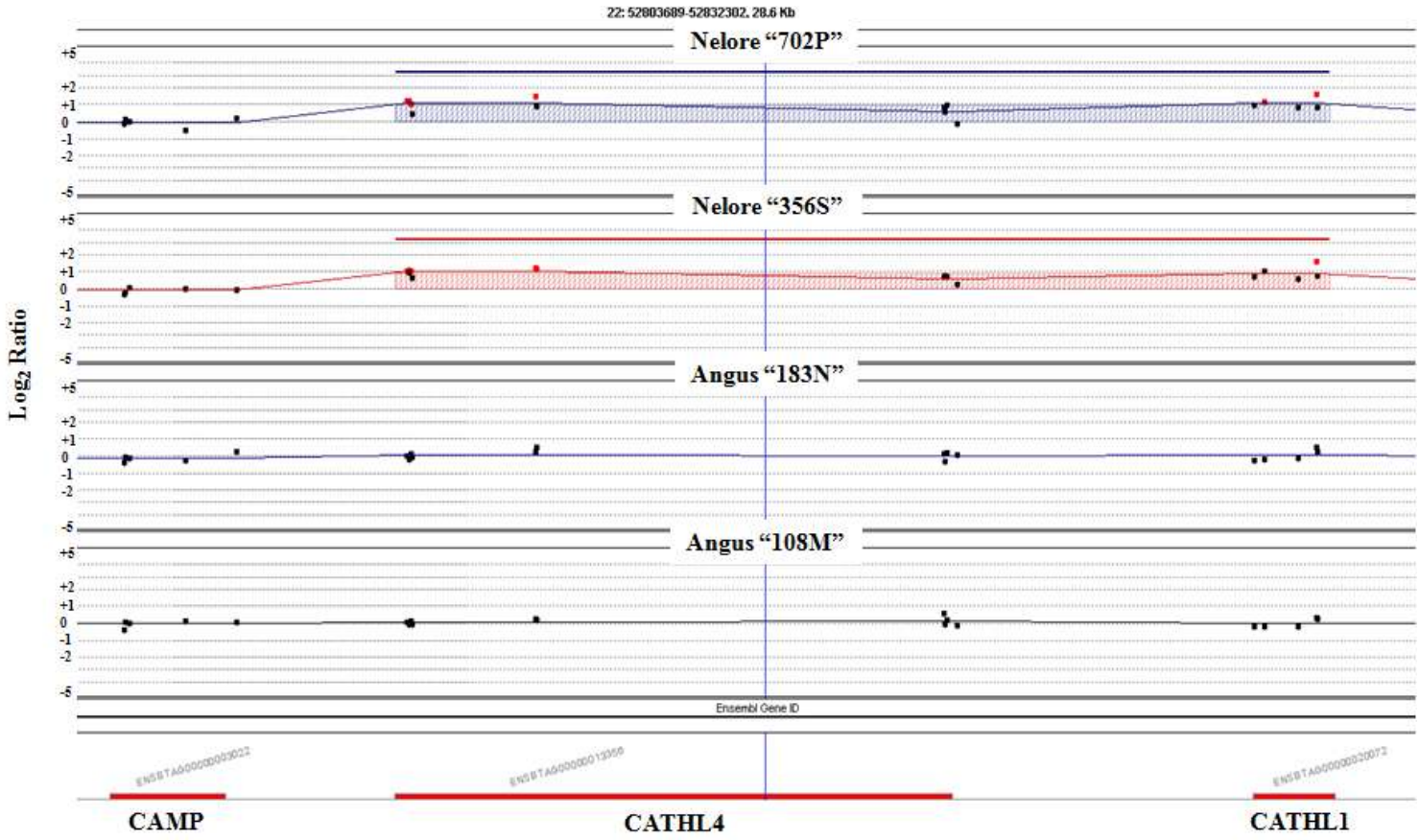

Fig. 3. Array CGH of CATHL4 for 2 Angus and 2 Nelore animals. Oligonucleotide probes were designed to span the bovine exome and align to the current Btau_4.0 assembly. Probes are shown as black and red dots (red dots signify significant change in fluorescence signal). Hybridization of DNAs were conducted overnight in a Hybridization Oven (Agilent Technologies). Then slides were washed and scanned with the SureScan Microarray Scanner (Agilent Technologies) was used to acquire array images. Fluorescence intensities and $\log_2$ ratio values were analyzed with the Agilent Genomic Workbench 5.0 software (Agilent Technologies).

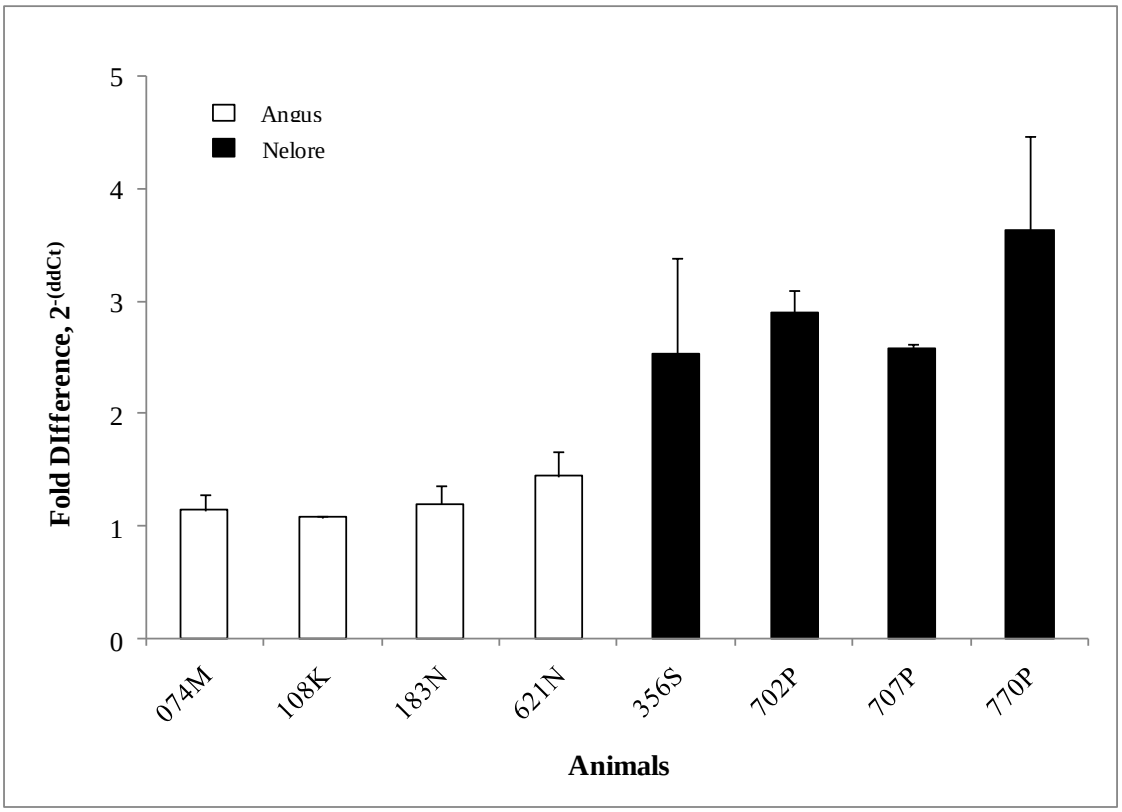

Fig. 4. Copy number variation of CATHL4 among Angus and Nelore cattle. qPCR was performed in duplicate using the comparative threshold cycle (C_T) method and SYBR Green chemistries. Sample C_T values were normalized to GAPDH C_T values and fold differences were determined by calculating $2^{-(ddCt)}$. Results are shown as means of duplicate reactions and error bars represent the standard error of means.

Copy Number Variation Among Domestic Cattle Breeds

A bovine breed panel consisting of 31 breeds and 93 individuals was screened for CNVs at the CATHL4 locus (Figure 5) by qPCR. Variability was observed both between and within each breed. One-way ANOVA was performed to analyze the variance of individuals' $2^{-(ddCt)}$ values within each breed, with the following results ($P \leq 0.01$): Brahman (P=4.92E-5, n=4), Brown Swiss (P=0.005, n=4), Braunvieh (P=6.09E-4, n=4), Charolais (P=2.12E-6, n=5), Holstein (P=0.001, n=4), Limousin (P=5.87E-4, n=3), Maine Anjou (P=0.002, n=5), Nelore (p=0.002, n=2), Santa Gertrudis (P=0.005, n=4), and Texas Longhorn (P=0.006, n=3). Table 6 classifies each breed as either *B. taurus taurus or B. taurus indicus* or a cross thereof. When an unpaired 2-tailed t-test compared crossbreeds' fold differences (average fold change = 4.62) to *B. taurus taurus* breeds fold differences (average fold change = 4.45) they were not considered statistically different (P=0.611, $P \leq 0.01$); when compared to *B. taurus indicus* fold differences (average fold change = 9.71), the p-value was 1.16E-9. An unpaired 2-tailed t-test was applied to determine if the 5-fold increase observed at the CATHL4 locus of *B. taurus indicus* breeds was were significantly different than *B. taurus taurus* breeds (P=1.17E-13, $P \leq 0.01$).

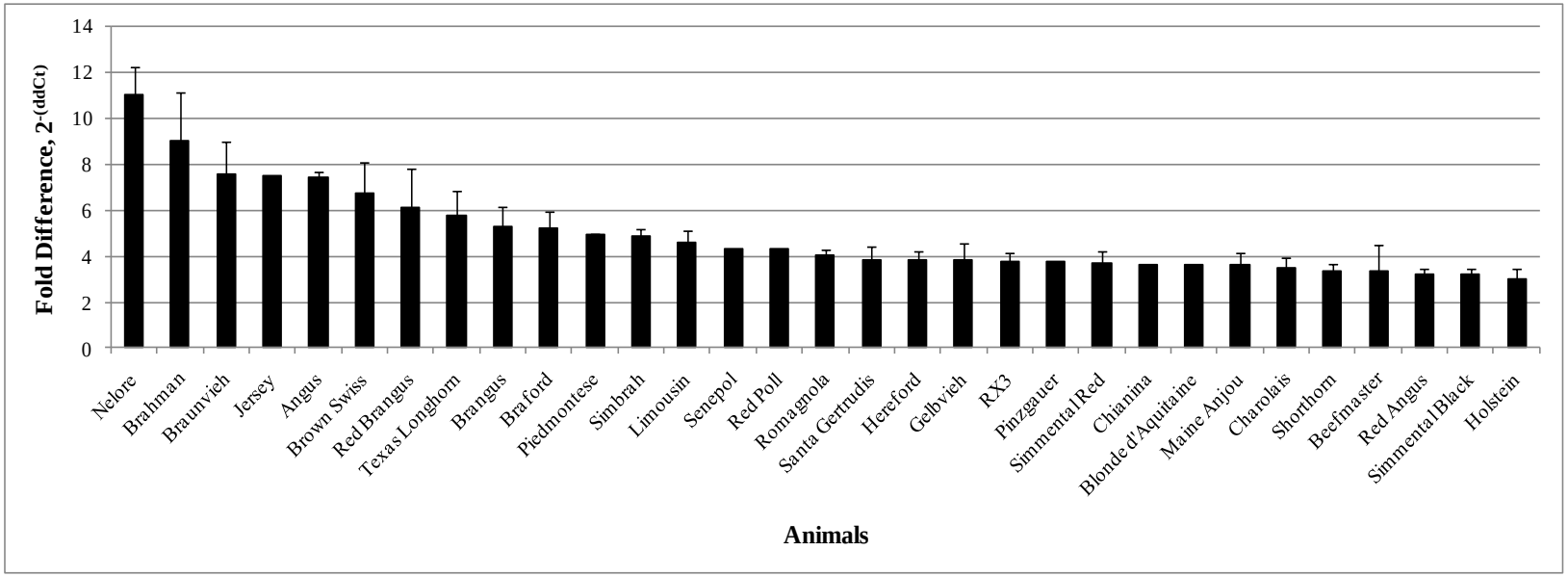

Fig. 5. Copy number variation of CATHL4 among domestic cattle breeds. qPCR was performed on the genomic DNA 93 animals of 31 breeds using the comparative threshold cycle (C_T) method and SYBR Green chemistries. Sample C_T values were normalized to GAPDH C_T values and fold differences were determined by calculating $2^{-(ddCt)}$. Results are shown as means of average C_T values of all animals of the same breed and error bars are shown as standard error of means.

Table 6. Classification of 31 domestic cattle breeds[a].

Bos taurus taurus	*Bos taurus indicus*	*Bos taurus* x *Bos indicus*
Angus	Brahman	Beefmaster
Angus (Red)	Nelore	Braford
Blonde d'Aquitaine		Brangus
Brown Swiss		Piedmontese
Braunvieh		Red Brangus
Charolais		Romagnola
Chianina		Santa Gertrudis
Gelbvieh		Senepol
Hereford		Simbrah
Holstein		
Jersey		
Limousin		
Maine Anjou		
Pinzgauer		
Red Poll		
RX3		
Shorthorn		
Simmental (Black)		
Simmental (Red)		
Texas Longhorn		

[a] Classifications based on breed information obtained from the Oklahoma State University Breeds of Livestock database (http://www.ansi.okstate.edu/breeds/cattle/).

Discussion

Previously aCGH was performed on DNA samples from 8 animals located at the Texas A&M University Department of Animal Science McGregor Research Center (McGregor tag IDs: "704M," "108M," "183N," "621N," "356S," "702P," "707P," and "770P"). Genomic DNA of 4 Angus and 4 Nelore animals were evaluated in a search for CNVs over the entire bovine exome (Doan *et al.*, unpublished data). CNVs were detected for numerous biologically relevant loci, including a region containing the bovine CATHL gene family. For this project, efforts focused on validating the CATHL4 locus CNV by qPCR. All 4 of the McGregor Nelore animals exhibited a 2-fold increase in relative copies to the 4 Angus animals. Additionally, a panel of 93 individuals from 31 domestic cattle breeds revealed that *B. taurus indicus* breeds showed approximately 5-fold higher relative copies than their taurine counterparts.

The breeds exhibiting the highest fold difference in CATHL4 CNVs were Nelore (~11-fold increase) and Brahman (~9-fold increase). Interestingly, Braunvieh, Angus, and Jersey breeds also ranked highly. The composite breeds Braford (Brahman-Hereford), Simbrah (Simmental-Brahman), Santa Gertrudis (Brahman-Shorthorn), and RX3 (Hereford-Holstein x Red Angus) exhibited a median fold difference compared to each founding breed in this panel (to the author's knowledge, no composite individuals were related to individuals of their respective founding breeds). However, Brangus' (Brahman-Angus) and Beefmaster's (Brahman-Hereford or Brahman-Shorthorn) fold changes did not fall between their respective founding breeds as expected. Given that the source of DNA for these animals was commercially available semen from a local

repository, it is possible that the animals used here were more heterogeneous in genetic background than their composite breed classification might indicate. Pedigrees are available for these animals and many of the sires of the same breed could be related or from the same ranch in this region. Evidence from sequence polymorphism studies in Chapter III revealed the Limousin bull shared many SNP genotypes with Brahman and Nelore animals. If haplotypes could be determined, the chromosomal contributions of each *Bos* subspecies might become more evident. This necessitates the need for evaluating more individuals in each breed from unrelated pedigrees and diverse regions throughout the country to confidently asses the CNV status of the CATHL4 locus.

In humans, approximately 9,000 genes have been mapped within or near regions of structural variation and CNVs are said to cover up to 12% of the genome [92]. With such large amounts of sequence implicated in CNVs, large-scale variation could contribute to normal phenotypic variability and disease susceptibility [50]. The findings presented here are among the first of many which confirm and characterize the complex structural variation within the bovine genome and lay the foundation for further investigations into the functional impact of CNVs and bovine disease processes. Genomic increases or decreases in copy number may not correspond to increases or decreases in mRNA or protein concentrations, so further validation is required before the full impact of the CATHL4 CNV can be known.

For decades, American cattlemen have incorporated indicine genetic backgrounds into their herds in the hopes of creating heartier cattle that are better adapted for warmer climates. However, little genetic evidence exists for these observed

differences. It was not until very recently that Murray *et al.* [93] concluded that both nucleotide diversity and haplotype diversity were higher within *B. taurus indicus* populations based on nearly 37,000 bp of sequence data. Many of the sequences used for their analysis were important to bovine immune response genes. Robust sequence analyses like those of Murray *et al.* combined with detailed investigations into the structural variation of genes like those in this study may begin to unveil a clearer picture of bovine immune response pathways.

CHAPTER V

VARIATION IN BOVINE CATHELICIDIN GENE EXPRESSION

Introduction

Copy number variations have become a target in the search for disease phenotypes and processes. Research has moved beyond simply detecting genomic CNVs to validating fold differences of expression and their impact on function. In livestock, whole genome microarrays have been used to evaluate gene expression profiles comparing resistant and susceptible phenotypes [49, 94, 95]. To date, four investigations have reported genomic CNVs in cattle of varying breeds [86-88, 96], but little has been reported regarding their functional impact. These previous studies necessitate verification of genomic CNVs, followed by verification of their respective transcripts, and finally their investigation into direct impacts on phenotypes.

The bovine CATHL4 AMP has been the subject of much research due to its small size and high tryptophan content. It has been tested against such clinically relevant pathogens as *Cryptosporidium parvum* and *Pneumocystis carinii*, both of which have been implicated in the morbidity of immunocompromised individuals, where it displayed broad-spectrum microbicidal activity [41]. It also inhibits the catalytic activity of HIV-1 integrase *in vitro* by directly binding to the integrase DNA [97]. Several studies have described bovine cathelicidin activity from neutrophils, but not non-myeloid cells [98, 99]. Therefore the focus of this study was the detection of fold differences of the CATHL4 transcript in bovine neutrophils.

Materials and Methods

Tissue Samples

Tissues samples from a freshly slaughtered steer were collected for mRNA isolation and detection of CATHL4 gene expression. Samples from esophagus, lung, tonsillar lymph node, and salivary gland were collected with sterile scalpels and biopsy punches and placed into RNase-free cryotubes. The tissues were immediately frozen in liquid nitrogen and kept at -80°C until processing.

For optimization of the neutrophil and RNA isolation protocols, several animals from the Texas A&M University Veterinary Medical Park were obtained as blood donors (AUP 2009-77). Neutrophils were isolated from whole blood immediately after collection. Up to 50 mL whole blood was collected via jugular venipuncture into sterile syringes with 10% EDTA-2Na solution. Neutrophils were then isolated by hypotonic lysis and gradient centrifugation. Briefly, in a 50 mL conical tube (Corning) 5 mL of whole blood was lysed with 18 mL ice cold sterile water for 20 seconds by gentle mixing, then lysed blood was divided into two 50 mL conical tubes. Immediately 30 mL of 2X phosphate buffered saline (PBS) was added to each tube to bring the osmolarity back to physiologic conditions. The tubes were centrifuged at 700 xg for 8 min, then the supernatant was carefully poured off. The pellet was resuspended in 6 mL cold 1X PBS and layered over 5 mL cold Histopaque-1077 (Sigma-Aldrich) in a 15 mL conical tube (Corning). The tubes were centrifuged at 700 xg for 8 min and the upper PBS layers and peripheral blood mononuclear cell (PBMCs) layers were carefully pipetted off with sterile transfer pipets and discarded. The sides of the tubes were wiped twice with sterile

cotton swabs to remove PBMCs. The pellet was washed once with 6 mL cold 1X PBS, spun at 700 xg for 8 min, and both tubes were combined and re-suspended in 2 mL cold 1X PBS. Cell counts and cell purity were verified with hemocytometer and trypan blue staining, with final concentrations averaging 6 x 10^7 cells/mL and >90% purity. Neutrophils were then transferred to 1.5 mL RNase-free microcentrifuge tubes (Eppendorf), spun for 10 sec at >14,000 xg, immediately flash frozen in liquid nitrogen, and stored at -80°C until processing.

RNA Isolation and cDNA Preparation

Total RNA was isolated and purified with the RNeasy Mini Kit (Qiagen) according to manufacturer's recommendations for both tissue samples and cell culture samples. All samples were subsequently treated with DNaseI (Invitrogen) to eradicate contaminating genomic DNA according to manufacturer's recommendations. RNA quantity was evaluated with a NanoDrop spectrophotometer (Thermo Scientific) and yields ranged from 150-350 ng/µl for tissue samples and 50-150 ng/µl for neutrophil pellets.

RNA samples were reverse transcribed into complementary DNA (cDNA) using Invitrogen's SuperScript III First Strand Synthesis Kit according to the manufacturer's recommendations. The maximum amount of total RNA available from each sample was added to each cDNA reaction and oligo(dT) primers provided in the kit were used to prime the reverse transcription reaction.

Quantitative Real-Time PCR of cDNA

Similar techniques to those used in qPCR of genomic DNA were used for qRT-PCR of cDNA. Primers were designed to span the fourth exon and the 3' UTR of CATHL4 to ensure distinction between possible genomic DNA. Again, Biosearch Technology's RealTimeDesign software (http://www.biosearchtech.com/realtimedesign) was used for primer design, and primer pairs were validated for specificity using the UCSC Genome Browser (http://genome.ucsc.edu/) set to the most current bovine genome assembly, Btau_4.0. SYBR Green MasterMix (Invitrogen) was used in conjunction with the ABI 7900HT Fast Real-Time PCR system for quantitative real-time PCR (qPCR) of the CATHL4 mRNA. Each reaction was carried out in RNase-free plasticware and contained the following: 1X final concentration SYBR MasterMix, 200 nM final concentration forward and reverse primers, 2 µl cDNA template, enough nuclease-free water to reach a final volume of 10 µl. Thermocycling parameters were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec, 60°C for 60 sec. Immediately following the last cycle a standard dissociation curve was performed to detect any primer-dimers in the PCR products. All PCR products were visualized by gel electrophoresis using 2% agarose gels stained with ethidium bromide. Using the relative comparative threshold cycle (C_T) method, duplicate C_T values were averaged and normalized against GAPDH for each assay [91]. Samples with C_T values that varied more than 0.5 were tested again. CATHL4 primers for cDNA were RT_CATHL4 F = 5'-GCTAATCTCTACCGCCTCCTG-3', RT_CATHL4 R = 5'-

GGCACACAGTCTCCTTCACC-3'and GAPDH primers were GAPDH F = 5'-GAAGGTGAAGGTCGGAGTC, GAPDH R = 5'-GAAGATGGTGATGGGATTTC).

Summary of Experimental Results

Quantitative Real-Time PCR of Tissues

In order to confirm the findings of Van Abel *et al.* [100] that CATHL4 expression should be highest in neutrophils, we isolated RNA from esophagus, lung, tonsillar lymph node, and salivary gland tissue samples from a freshly slaughtered steer. Previously, whole blood had been collected from an animal from the Texas A&M University Veterinary Medical Park and RNA was isolated from both neutrophils and PBMCs. qPCR was performed in duplicate using the comparative threshold cycle (C_T) method and SYBR Green chemistries. Sample C_T values were normalized to GAPDH C_T values and fold differences were determined by calculating $2^{-(ddCt)}$. Even though the samples were from different animals, neutrophils showed a 5-fold increase in relative copies of CATHL4 transcripts compared to PBMCs (P=0.014, unpaired 2-tailed t-test comparing neutrophil to PBMC fold difference), and a range of 5- to 8-fold differences in relative copies of CATHL4 transcripts compared to the other tissue samples (Figure 6).

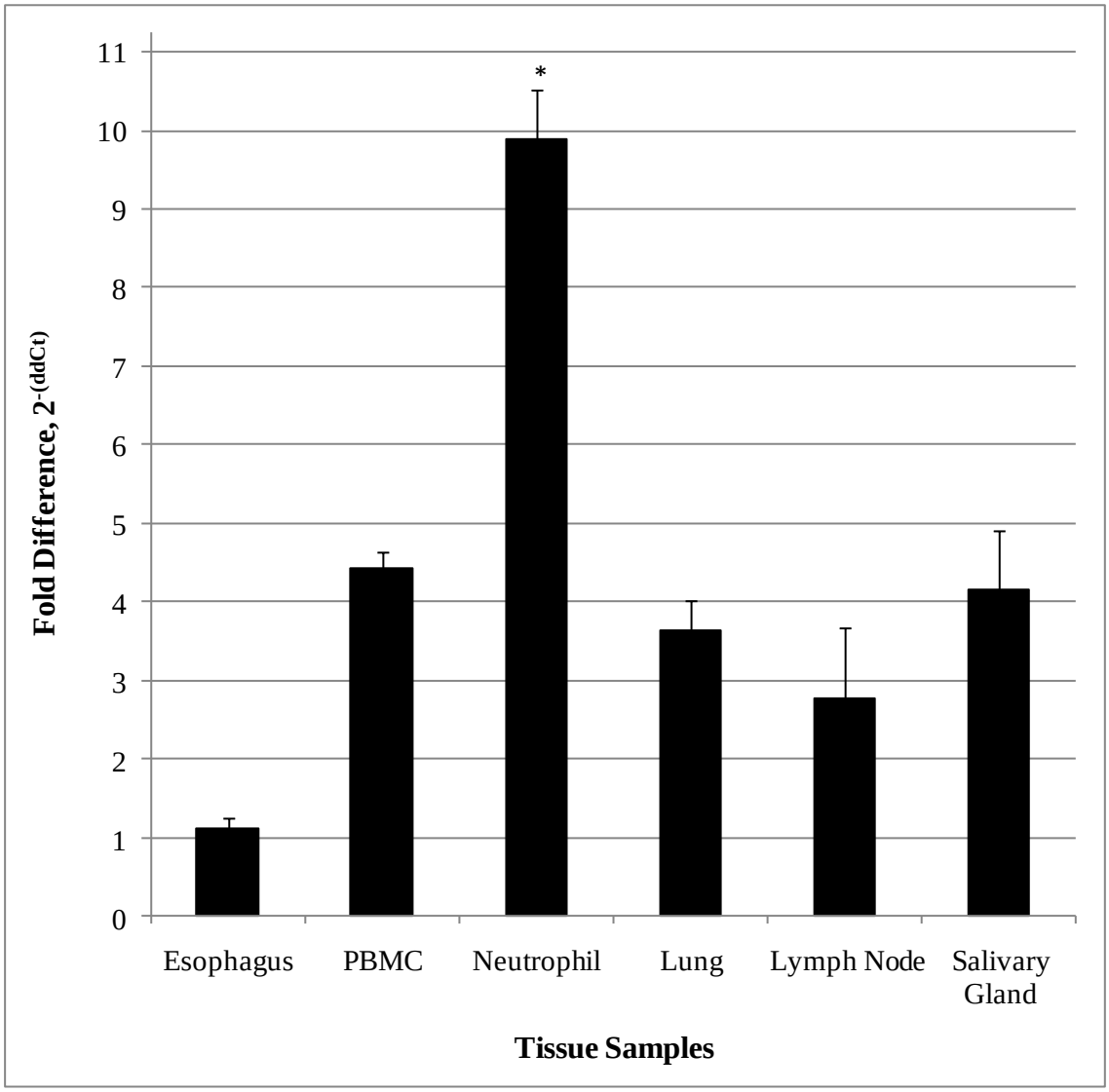

Fig. 6. Variation of CATHL4 gene expression in bovine tissue samples. qPCR was performed using the comparative threshold cycle (C_T) method and SYBR Green chemsitries. Sample C_T values were normalized to GAPDH C_T values and fold differences were determined by calculating $2^{-(ddCt)}$. Results are shown as means of duplicate reactions and error bars represent the standard error of means. *$P \leq 0.05$ for unpaired 2-tailed t-test comparing neutrophil to PBMC fold increases.

Quantitative Real-Time PCR of CATHL4 Transcripts of Angus and Nelore Animals

Neutrophils and RNA were isolated from whole blood that was collected from the eight animals selected for this study from Texas A&M University's Department of Animal Science McGregor Research Center. qPCR was performed on cDNA in duplicate using the comparative threshold cycle (C_T) method and SYBR Green chemistries (Figure 7). Sample C_T values were normalized to GAPDH C_T values and fold differences were determined by calculating $2^{-(ddCt)}$. One animal, "Nelore 356S" was particularly temperamental in the chute and several attempts were made to collect blood. Evaluation by qPCR revealed an approximate 400-fold increase in CATHL4 expression so it was not included in this analysis. While "Nelore 707P" exhibited a 5-fold increase in CATHL4 gene expression, when an unpaired 2-tailed t-test was performed to compare Angus to Nelore fold-differences, the two groups were not significantly different ($P=0.011$, $P\leq0.01$).

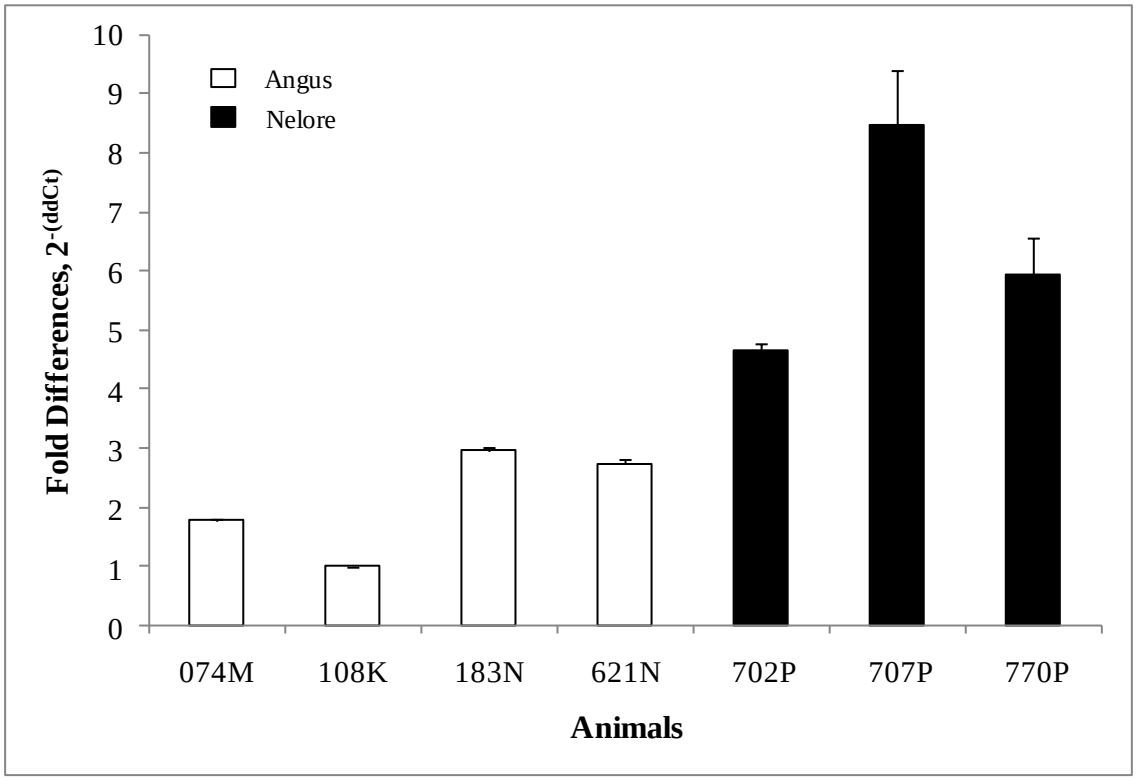

Fig. 7. Fold differences in Angus and Nelore CATHL4 gene expression. RNA was isolated from the neutrophils of Angus and Nelore animals. qPCR was performed on cDNA using the comparative threshold cycle (C_T) method and SYBR Green chemistries. Sample C_T values were normalized to GAPDH C_T values and fold differences were determined by calculating $2^{-(ddCt)}$. Results are shown as means of duplicate reactions and error bars represent the standard error of means.

Discussion

Based on previous findings that genomic CNVs exist for the bovine CATHL4 gene, the focus of this study was to determine what impact genomic structural variation may have on CATHL4 gene expression. Neutrophils were isolated from whole blood from 4 Angus (tag IDs: "074M," "108K," "183M," "621N") animals and 4 Nelore (tag IDs: "356S," "702P," "707P," "770P") animals from the Texas A&M University Department of Animal Science McGregor Research Center. Total RNA was extracted and cDNA was screened by qPCR and SYBR Green chemistries. The comparative threshold cycle (C_T) method was used for analysis; GAPDH C_T values were subtracted from each sample C_T value (ΔC_T), which was then subtracted from the highest ΔC_T value ($\Delta\Delta C_T$). Fold difference was calculated as $2^{-(\Delta\Delta CT)}$. Based on average $2^{-(\Delta\Delta CT)}$ values for each breed, Nelore cattle showed an approximate 2- to 5-fold increase in expression of CATHL4 over the Angus. One individual, "Nelore 356S" was temperamental in the chute during blood collection and was calculated to have an approximate 400-fold increase in expression. The transport time from the McGregor center back to Texas A&M University is approximately 90 min, which could allow for neutrophil activation if the blood had become contaminated with bacteria and other stress hormones associated with confinement in the chute. Neutrophil recruitment and activation could skew baseline results for CATHL4 expression. The other Nelore animals exhibited an average 4-fold increase in CATHL4 gene expression but were not significantly higher than the Angus averages.

Recently, the regulation of CATHL gene expression has been extensively studied in humans. Several studies have demonstrated that human CATHL (LL-37) is induced by the hormone 1,25-dihydroxyvitamin D_3 (vitamin D_3). Activation of human toll-like receptors (TLR) 2/1 and TLR4 induces the expression of vitamin D_3 via the α-hydroxylase CYP27B1 [101]. Then, vitamin D_3 signals through the vitamin D_3 receptor (VDR) transcription factor, which in turn binds to vitamin D_3 response elements (VDREs) in the LL-37 promoter region [102]. Induction of cathelicidin by vitamin D_3 was confirmed in purified keratinocytes, monocytes, neutrophils, and other human cell lines [102] but not in murine bone marrow cells [103]. Gombart *et al.* [103] showed that sequence homology did exist in the promoter regions of human, chimpanzee, rat, dog, and mouse, but the SINE found in humans and chimpanzee which contains the VDRE was absent in the mouse genome.

The results of this study are strictly limited to a small number of animals and the extreme fold change observed in Nelore "356S" was not included in the final analysis, so caution should be exercised when making broad conclusions regarding the state of CATHL4 expression in bovine neutrophils. Previous studies suggest that in as little as 4 hours LL-37 expression in human neutrophils is greatly increased in the presence of bacterial lipopolysaccharide (LPS) [102]. Little is known about the regulation and induction of bovine CATHL gene expression, but it is likely that qPCR quantitation of bovine CATHL4 gene expression is greatly affected by the amount of time neutrophils are exposed to bacteria. The conditions for collecting neutrophils in future experiments

should be strictly regulated and samples should be processed immediately to minimize these effects.

CHAPTER VI

COLORIMETRIC ASSAY FOR NEUTROPHIL KILLING CAPACITY

Introduction

Peripheral blood neutrophils represent a first line of defense for cattle against invading pathogens. They mature in the bone marrow, differentiate, and then are released in to the bloodstream. Neutrophils are considered granulocytes for the numerous granules found in the cytoplasm. These are specialized lysosomes which contain acid hydrolases and AMPs. When recruited to sites of infection, neutrophils engulf the microbe into a membrane-bound phagosome. The phagosome fuses with granules (phagolysosome) wherein the microbes are exposed to AMPs, enzymes, and reactive oxygen species (ROS) [104]. Neutrophils represent a large portion of the phagocytic cells found in bovine blood and evaluation of their bactericidal activity could be important in determining an animal's immune status and overall health [105]. Additionally, it has been established that the mature AMP of CATHL4 can be found in the cytoplasmic granules of circulating neutrophils [106], [100], [107]. Therefore, determination of neutrophil bacterial killing capacity may be a valuable tool for evaluating the status of animals' immune systems during disease.

BRD is considered to be one of the costliest diseases of the American cattle industry, and has been extensively researched since the late 1800s [108]. Often, *Mannheimia haemolytica* (Gram-negative) and *Pasteurella multocida* (Gram-negative) have been implicated as the bacterial agents of BRD based on clinical occurrences [109],

vaccination trials [110] , and serologic surveys [111]. These two bacterial strains, along with *Staphylococcus aureus* (Gram-positive), and *Salmonella typhimurium* (Gram-negative), were chosen for bacterial challenge.

Here, a simple colorimetric assay was used to determine neutrophil bactericidal activity. The tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is converted by bacteria intracellularly into purple formazan and is directly proportional to viable bacterial cell number [105]. Neutrophil killing capacity can be measured by colorimetric determination of formazan production by live bacteria.

Materials and Methods

Bacterial Strains and Growth Conditions

Stock strains of *S. aureus, S. typhimurium, P. multocida,* and *M. haemolytica*, were plated and incubated at 35°C overnight. Isolated bacterial colonies were transferred with a sterile toothpick into a 15 mL glass tube with aeration top containing 5 mL LB broth and incubated overnight at 35°C on a shaker. The bacteria were grown to a concentration of $1x10^9$ CFU/mL, then 1 mL was transferred to a 1.5 mL microcentrifuge tube (Eppendorf) and centrifuged for 2 min at 14,000 xg. The supernatant was decanted and the bacteria were washed with 1 mL RPMI-1640 (GIBCO, Invitrogen) supplemented with 5% fetal bovine serum (FBS) (GIBCO, Invitrogen) and centrifuged for 2 min at 14,000 xg. All RMPI-1640 was supplemented with 5% FBS unless otherwise noted. The supernatant was decanted and the bacteria were

resuspended in 1 mL RPMI. 500 µl of the bacterial suspension was added to 4.5 mL RPMI for a final concentration of $1x10^8$ CFU/mL.

In order to be a recognizable target for neutrophils, all bacteria were opsonized prior to use in the MTT assay. Briefly, whole bovine blood serum was heat inactivated by incubation for 30 min in a 56°C water bath. In a 3 mL glass vacutainer, 1.975 mL of bacteria suspension were combined with 25 µl of heat inactivated serum and incubated for 30 min at 37°C while rotating.

Bovine Neutrophil Isolation

The members of the Lawhon laboratory previously optimized this isolation protocol for use in the MTT assay. Therefore this isolation protocol is slightly different from the protocols described above. Whole blood was collected from 8 animals (AUP#) from the Texas A&M University Department of Animal Science McGregor Research Center (tag IDs: "704M," "108M," "183N," "621N," "356S," "702P," "707P," and "770P") via jugular venipuncture into six ACD venous blood collection tubes (Becton, Dickinson and Co.) and put immediately on ice. Each tube was mixed by inversion, and then centrifuged for 25 min at 1,000 xg, 4°C. Plasma, buffy coats, and the top third of the RBC layer were removed by sterile transfer pipettes; the remaining RBCs from 3 ACD tubes were combined (~15 mL) in to one 50 mL conical tube (Becton, Dickinson and Co.). RBCs were hypotonically lysed with 15 mL of ice cold sterile water and mixed by inversion for 20 sec. Immediately 3.75 mL of ice cold 5X PBS was added and gently inverted. Cells were pelleted after a 10 min centrifugation at 250 xg, 4°C and the

supernatant was carefully discarded. Cell pellets were washed with 10 mL ice cold 1X PBS, vortexed, and centrifuged for 3 min at 500 xg, 4°C. Hypotonic lysis was repeated once as necessary, and final cell pellets were washed twice with 10 mL ice cold RPMI, vortexed, and centrifuged for 3 min at 500 xg, 4°C for the first wash and 450 xg, 4°C for the second wash. The supernatants were decanted and cells were resuspended in 2 mL RPMI. Cell counts and viability were calculated automatically with a Cellometer Auto T4 (Nexcelom Bioscience), and then diluted to a final concentration of $1x10^7$ cells/mL.

MTT Assay

The MTT assay used here is based on the protocols outlined by Stevens in 1993 [112] and 1991 [105]. All assays were completed in triplicates, both biologically (triplicate microplates) and technically (triplicate wells). A standard curve was included on each 96-well microplate (Corning, Sigma-Aldrich) and was completed as follows: 50 µl of bacteria were added to the first well ($5x10^7$ bacteria, representing 0% killing and bacterial negative control), 35µl to the second well ($3.5x10^7$ bacteria, representing 30%), 20 µl to the third well ($2x10^7$ bacteria, representing 60% killing), and 5 µl to the fourth well ($5x10^6$ bacteria, representing 90% killing); enough RPMI was added to bring the final volume of all wells in the standard curve to 100 µl (50 µl, 65 µl, 80 µl, and 95 µl, respectively). Additionally, a neutrophil negative control was included in each assay as follows: 50 µl of $1x10^7$ neutrophils/mL suspended in RPMI were combined with 50 µl extra RMPI to bring the final volume up to 100 µl.

All other samples received ten bacteria/neutrophil by adding 50 µl opsonized bacteria to treatment wells, followed by 50 µl of $1x10^7$ neutrophils/mL suspended in RPMI. The microplates were incubated for 30 min at 37°C and 5% CO_2. Then, all neutrophils were lysed by adding 50 µl 0.2% saponin and allowed to sit at room temperature for 5 min. Next, 50 µl of MTT (2 mg/mL, Sigma-Aldrich) was added to all wells and incubated for 10 min at room temperature. Microplates were then centrifuged for 10 min at 1,800 xg to pellet the formazan-laden bacteria. Supernatants were decanted by gently blotting the microplates upside down on absorbent paper, and then 150 µl dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to all wells to solubilize the formazan. The microplates were vigorously agitated for 10 min and 50 µl 1X PBS was added before absorbance was immediately measured at 560 nm on a Epoch Microplate Spectrophotometer (BioTek).

Summary of Experimental Results

Standard Curves of Bactericidal Activity

Figure 8 contains the standard curves of bactericidal activity that were used to measure the killing of bacteria after 30 min incubation of 10 bacteria/neutrophil. Production of formazan from MTT by *S. aureus*, *S. typhimurium*, *M. haemolytica*, and *P. multocida* was related and highly correlated ($R^2 \geq 0.9763$ and $P \leq 0.001$ by linear regression and one-way ANOVA) with numbers of live bacteria. For each bacterial species, the challenge dose of $5x10^7$ bacteria/microplate well, and dilutions containing a 30% ($3.5x10^7$/well), 60% ($2x10^7$/well), and 90% ($5x10^6$/well) decrease in numbers of

bacteria could be accurately quantitated by measuring bacterial production of formazan from MTT.

Bovine Neutrophil Bactericidal Activity

Neutrophils of 4 Angus (tag IDs: "074M," "108K," "183M," "621N") and 4 Nelore (tag IDs: "356S," "702P," "707P," "770P") animals were incubated for 30 min with 10 bacteria/neutrophil and subsequent bacterial conversion of MTT to formazan was measured by absorbance at 560 nm. Angus neutrophils displayed 40.2% and 8.6% killing capacity against *S. aureus* and *S. typhimurium*, respectively, while Nelore neutrophils displayed 34.8% and 3.3% killing capacity, respectively (Figure 9). However, Angus neutrophils displayed only 39.6% and 21.2% killing capacity against *M. haemolytica* and *P. multocida*, respectively, while Nelore neutrophils displayed 61.9% and 26.0% killing capacity, respectively (Figure 9). When unpaired 2-tailed t-tests were performed comparing Angus to Nelore, none of the killing capacities observed here were significant ($P=0.049$, $P\leq0.01$).

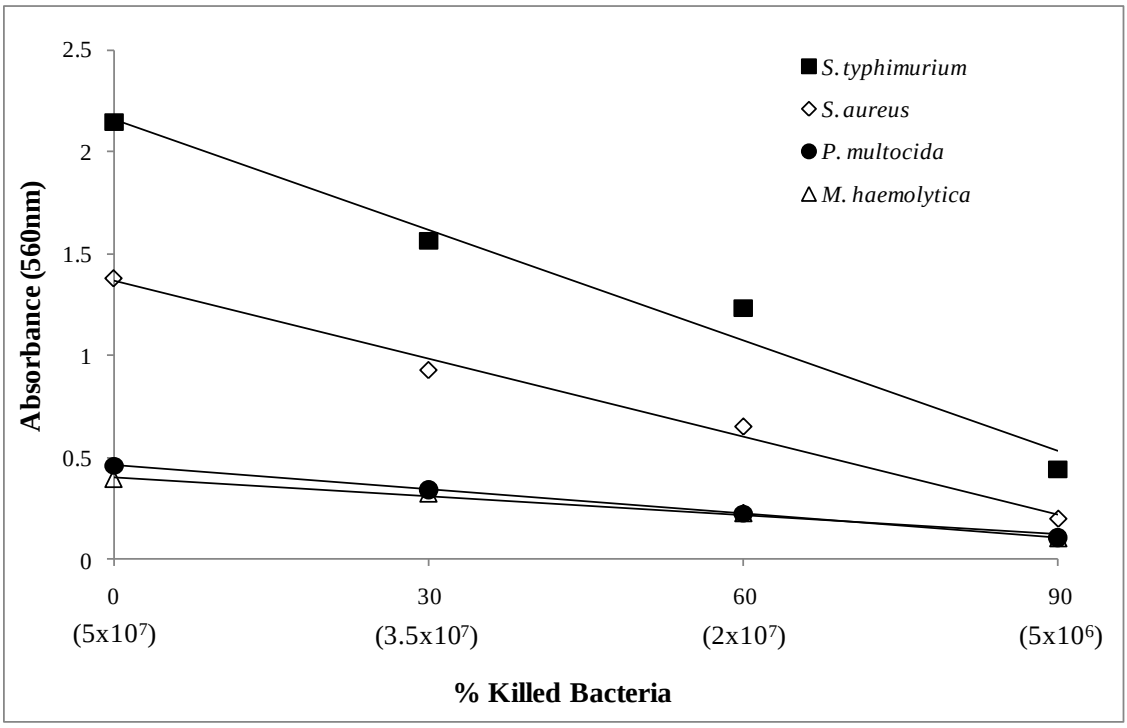

Fig. 8. Standard curves of bactericidal activity. MTT was used to measure numbers of live bacteria. *S. aureus*, *S. typhimurium*, *M. haemolytica*, and *P. multocida* at $1x10^7$ bacteria/mL in RPMI containing 5% FBS were diluted 0, 30, 60, and 90%. Results are presented as means of both biological and technical triplicates. For all standard curves, $R^2 \geq 0.9763$ and $P \leq 0.001$ by linear regression and one-way ANOVA. Values in parenthesis indicate number of bacteria per microplate well.

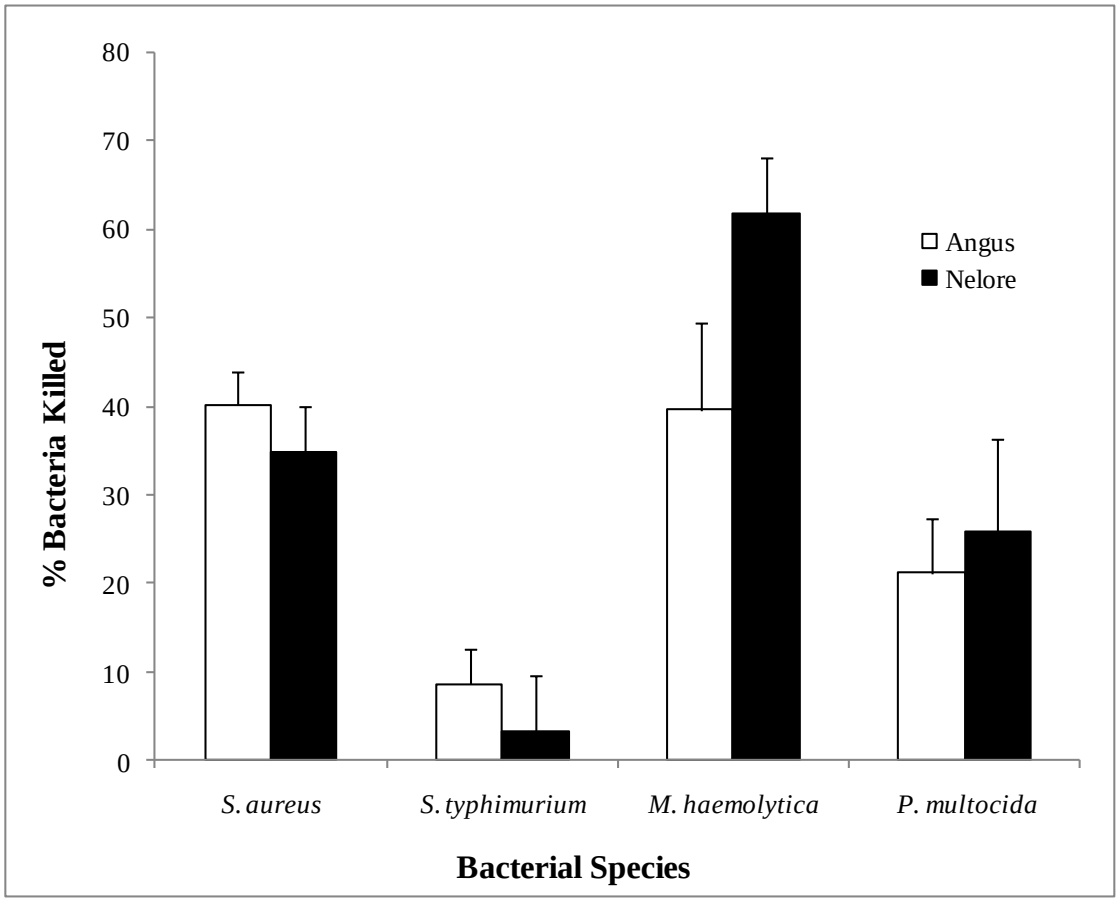

Fig. 9. Killing capacity of Angus and Nelore neutrophils. Neutrophils were incubated with 10 bacteria/neutrophil (*S. aureus*, *S. typhimurium*, *M. haemolytica*, *P. multocida*) for 30 min, then 2 g MTT/mL were added to measure the number of surviving bacteria. Results are displayed as means of biological and technical triplicates with standard error of means.

Discussion

This colorimetric MTT bactericidal assay is based on the principle that bacteria convert MTT to formazan in direct proportion to live cell counts, and the amount of formazan produced by live bacteria is one measure of bovine neutrophil killing capacity. Levels of formazan produced by remaining bacteria were compared to a standard curve produced by known numbers of bacteria, and the percentage of bacteria killed by neutrophils was extrapolated from the standard curve. All species of bacteria used here have been previously shown to convert MTT to formazan efficiently [105].

Lymphocytes from 8 animals from the Texas A&M University McGregor Research Center (tag IDs: "074M," "108K," "183M," "621N," "356S," "702P," "707P," "770P") were chosen for a colorimetric MTT bactericidal assay to determine variability in neutrophil killing capacity *in vitro*. These animals were previously genotyped for genomic CNVs at the CATHL4 locus. Whole blood was collected via jugular venipuncture and neutrophils were isolated for bacterial challenge. *Staphylococcus aureus*, *Salmonella typhimurium*, *Mannheimia haemolytica*, and *Pasteurella multocida* bacterial strains were used due to their experimental reliability and relevancy to Bovine Respiratory Disease (BRD). For *S. aureus* Angus showed a killing capacity of 40.2% and Nelore showed 34.8%; for *S. typhimurium* Angus showed 8.64% and Nelore showed 3.31%; for *M. haemolytica* Angus killing capacity was 39.6% and Nelore was 61.8%; and finally, for *P. multocida* Angus showed 21.2% and Nelore showed 25.9%. These results suggest not correlation between genomic CATHL4 CNVs and the amount of CATHL4 AMP produced, but the sample size is quite small. In order

to determine the true impact CATHL4 CNVs have on any immune-related phenotype, positive validation of the association between genomic copy number changes, mRNA fold differences, and changes in final protein concentration should be thoroughly investigated with larger sample sizes and more robust detection techniques (TaqMan qPCR, Western blotting, immunohistochemical staining, ELISA, etc).

While *M. haemolytica* and *P. multocida* have often been implicated as the bacterial agents of BRD, the disease is likely the result of numerous environmental offenders, including viral infection, stress, and change in climate during shipping and transport. To date, little research has investigated the role of antimicrobial peptides in BRD. Preliminary findings presented here may encourage future in-depth investigations into the host-pathogen interactions with regard CNVs of innate immune system genes.

CHAPTER VII

CONCLUSIONS

Discussion

The peptides discussed here are proficient in killing microbes independently but often show improved microbicidal activity in the presence of other AMPs. Transgenic mice expressing porcine CATHL PR-39 showed enhanced activity of the native murine CATHL, (mCRAMP), against Group A *Streptococcus* (GAS) *in vitro* [113]. This suggests that addition of distinct, xenobiotic CATHL proteins may confer increased resistance to bacterial infection, and these findings provide a basis for future studies aimed at determining whether the bovine CATHLs synergistically enhance human immune defense. Subsequently, the bactericidal killing capacity of bovine neutrophils genotyped with higher numbers of CATHL4 copies may be in partly due to an expansion of copies of the entire CATHL region. If expanded structural variation is confirmed in the future, the increased resistance to environmental pathogens of *B. taurus indicus* animals could be attributed to increased CATHLs of all structural classifications, which in turn synergistically defend the host at an increased frequency and efficiency.

Since many of the polymorphisms presented in Chapter III are either synonymous or intronic and do not appear to confer a change in protein sequence, it is important to explore the impact they may have in overall gene function. Most amino acids are encoded by several codons that usually only differ in the third nucleotide. These alternative synonymous codons are not used at equal frequencies, which give rise

to two phenomena: variations of codon employment patterns among species; and biased codon usage in highly expressed genes [114]. The effects of synonymous mutations and codon bias can appreciably impact several post-translational modifications and overall gene function.

One common form of post-translational modification is alternative exon splicing. In humans it was discovered that intronic regions (and their synonymous SNPs) surrounding splice sites are more conserved than those surrounding constitutive exons; evidence also exists for purifying selection of synonymous SNPs in exonic splice enhancers (ESEs) [115]. Synonymous mutations also effect changes in the secondary structures of mRNAs and tertiary structures of proteins. Recent studies focus on the consequences synonymous mutations and alternative splicing in this context. Conclusions suggest that the stability of stem-loop mRNA structures vary greatly; and the recruitment of rare codons (due to synonymous mutations) could lead to pausing in translational machinery, allowing time for the protein to find new structures [116]. Lastly, many miRNA binding sites occur in intronic regions and regulate mRNA transcripts by sense-antisense pairing. The need for correct pairing between mRNA and miRNA likely imposes evolutionary constraints on the binding sequence, and therefore constrains the evolution of both non-synonymous and synonymous mutations within that sequence [117].

In bone marrow, there are reported splice variants in the human CATHL AMP, hCAP18, which may have effects on maturation, emigration, and microbicidal activity [118]. Identification of a potent repressor in the 5'-UTR, an upstream enhancer, and 2

GT-boxes that positively regulate hCAP18 promoter activity demonstrate complex regulation of the human CATHL [118]. While currently no findings have been reported for bovine CATHL regulatory elements or splice variants, the findings regarding human CATHL regulation are a basis for future investigations into upstream DNA segments which may help regulate the large bovine CATHL family.

Final Conclusions

Chapter II presented a high-resolution radiation hybrid map comprised of 7 markers surrounding the bovine CATHL gene cluster, previously placed at the distal end of BTA22. The final framework map supports overall gene order and places a currently unannotated duplicate gene with 99% sequence similarity to CATHL4 on the map. Next, in Chapter III, sequence polymorphisms uncovered in 4 bovine CATHL genes revealed 60 SNPs and 5 indels. None of the SNPs were in exon IV, the exon which codes for the mature functional AMP. Based on previous work by Doan *et al.*, structural variability in the form of CNVs was detected in the CATHL region. Here, the status of genomic CNVs for bovine CATHL4 was confirmed by qPCR of genomic DNA from 4 Angus and 4 Nelore animals, chosen for their diverse genetic backgrounds. A 2-fold increase in genomic copies of CATHL4 was observed in Nelore animals. In order to determine the impact of genomic CNVs of CATHL4 on gene expression, RNA was isolated from neutrophils from the same 8 animals. Quantitative RT-PCR was performed on cDNA and a 2- to 5-fold increase in expression in Nelore animals was observed, though variable environmental factors make it difficult to conclusively

determine the changes in expression that result from genomic CNVs. Finally, a colorimetric MTT assay was performed to determine the bacterial killing capacity of neutrophils isolated from the same 8 animals. Neutrophils were incubated with *Staphylococcus aureus, Salmonella typhimurium, Mannheimia haemolytica,* and *Pasteurella multocida* bacterial strains; Angus animals showed a 6% higher killing capacity than Nelore for S. *aureus,* a 5% higher killing capacity for *S. typhimurium,* a 22% lower killing capacity for *M. haemolytica*, and a 4% lower killing capacity for *P. multocida*. The findings presented here characterize the bovine CATHL gene family in physical gene order, in sequence variability and in genomic structural variability. Preliminary experiments into the expression patterns and functionality of bovine CATHL4 provide a foundation for future studies into CATHLs' role in maintaining healthy, immunocompetent hosts.

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